Report of Sabbatical Leave

September 6, 1982 to January 31, 1983

Presented to the

Board of Trustees

Mount San Antonio College

Walnut, California

Betty B. Ward

Instructor, Biology Department

November 4, 1983

ACKNOWLEDGEMENT

I wish to express my appreciation to the Mount San Antonio College District for providing this opportunity for study, travel and personal growth. It has broadened my knowledge and experiences and has provided information invaluable for the enrichment of course material and the writing of a Microbiology laboratory manual.

The concept of Sabbatical Leaves is sincerely appreciated and the benefit to the students and the college is recognized.

MT. SAN ANTONIO COLLEGE
Salary and Leaves Committee

MT. SAN ANTONIO COLLEGE
DEC 30 AM 10:55
PERSONNEL OFFICE

APPLICATION FOR SABBATICAL LEAVE

Name of Applicant Ward (Last) Betty (First) Beasley (Middle)

Address 447 South Walnut (Street) Brea (City) 92621 (Zip)

Employed at Mt. San Antonio College beginning September (Month) 1970 (Year)

Dates of last sabbatical leave:

From _____ (Month) _____ (Year) To _____ (Month) _____ (Year)

Department Biology Department Division Natural Sciences

Length of sabbatical leave requested:

One semester X

Fall X Spring _____

Two semesters _____

Administrative _____

Purpose of sabbatical leave:

Study _____

Travel _____

Independent Study or Research _____

Combination (specify) X Independent Study and Travel

Effective dates for proposed sabbatical leave:

From September 6, 1982 To January 31, 1983

and (if needed)

From _____ To _____

Attach a comprehensive, written statement of the proposed sabbatical activity(ies) including a description of the nature of the activity(ies), a timeline of the activity(ies) an itinerary, if applicable, the proposed research design and method(s) of investigation, if applicable.

Attach a statement of the anticipated value and benefit of the proposed sabbatical activity(ies) to the applicant, his/her department or service area, and the College.

Any change or modification of the proposed sabbatical activity(ies) as evaluated and approved by the Salary and Leaves Committee must be submitted to the Committee for reconsideration.

Betty A Ward
Signature of Applicant

November 30 1981
Date

Applicant's Name Betty B. Ward

The acknowledgment signatures reflect awareness of the sabbatical plan for the purpose of personnel replacement. Comments requested allow for recommendations pertaining to the value of the sabbatical leave plan to the College. Applicants must obtain the signatures of acknowledgment prior to submitting application to the Salary and Leaves Committee.

ACKNOWLEDGMENT BY THE DEPARTMENT/DIVISION

Signature of Department Chairperson William R. Dawkins Date 11-30-81

Comments:

Signature of Division Chairperson Albert W. Bruno Date 11/30/81

Comments:

ACKNOWLEDGMENT BY THE OFFICE OF INSTRUCTION

Signature of Vice President/Asst. Superintendent Instructional & Student Services Joseph M. Bajorski Date 11-30-81

Comments:

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FINAL ACTION BY THE SALARY AND LEAVES COMMITTEE:

✓ Recommend approval to the Board of Trustees

_____ Not recommend approval to the Board of Trustees

Warren A. Collins 6-28-82
Signature - Chairperson, Salary and Leaves Committee Date

John O Rourke 11/29/83
Signature - Authorized Agent for the Board Date

myw
10/13/81

Major advances in Microbiology and related applied sciences necessitate the revision and enrichment of both the Microbiology lectures and laboratories. The advanced technologies of the research laboratories have greatly increased our knowledge in such areas as microbial cytology, physiology, ecology, pathology and behavior and altered the methodologies of applied science laboratories. As a result, considerable additional time is required for study, assessment, and evaluation in order to provide students with the most relevant concepts, methodologies and technological skills basic to their educational needs, interests and future careers.

I propose a one semester sabbatical leave for the purpose of revision and enrichment of lectures and the development of a Microbiology laboratory manual for use by Mt. San Antonio students. My plan involves:

1. independent study at the University of California and California State University at Fullerton specifically in the areas of microbial cytology and genetics including genetic engineering.
2. travel consisting of visits and consultations at campuses, hospitals, medical and bacteriological laboratories and clinics, research centers, water and sewage plants, food processing industries, museums, Public Health Depatments and Food and Drug Administration offices. Included, as time and opportunity permit, are the Food and Drug Administration in Washington, D.C., Center for the Control of Communicable Diseases in Atlanta, Georgia, Radiation Medicine Laboratories* in Oakridge, Tennessee, the N.A.S.A. Space Centers* in Virginia and Florida and Rockwell Space Division* in Palmdale, California.

*Permission to visit already granted.

I anticipate that my proposed sabbatical project will be beneficial in the following ways:

1. Such studies, travel and consultations will broaden my knowledge and experience and increase my value as an instructor to my students and the college.
2. In that microorganisms are important issues of social concern, eg. the exploration of space and inner space, the potentials and hazards of genetic manipulation, ecological and sanitary concerns and the cycles and balances of nature, and have provided the most useful models and experimental subjects for the study of life processes, eg. genetics at the molecular level, biosynthetic pathways, metabolic control mechanisms, etc., such information I gain would most certainly generalize and be useful in other Biology courses offered by my department.
3. It will provide information invaluable for the revision and enrichment of Microbiology and Biology subject material.
4. It will form the basis for the development of a Microbiology laboratory manual involving the use of those advanced tools and technological procedures of research and applied sciences laboratories which are most relevant to the specific career-oriented laboratory skills, experiences and training required by Microbiology students. In that our course is the only Microbiology course to be taken by most of our students, it is imperative that it offer the greatest opportunity to acquire knowledge through precise, direct observations, accurate logical reasoning and relevant, advanced, career-oriented experimentations.

MT. SAN ANTONIO COLLEGE

1100 NORTH GRAND AVENUE • WALNUT, CALIFORNIA 91789

Telephone: (714) 594-5611

January 29, 1982

Mrs. Betty Ward
Biological Sciences
Campus

Dear Mrs. Ward:

The Salary and Leaves Committee has completed the review and evaluation of sabbatical leave applications for the 1982-83 school year. I am pleased to inform you that the Committee will recommend that the Board of Trustees approve your sabbatical at the February, 1982 Board Meeting. You will subsequently be informed of the Board's action.

As explained in the sabbatical leave orientation meeting and in the published materials developed by the Committee, the evaluation of sabbatical applications was based upon established criteria and thorough review by the Committee.

You are respectfully reminded that, upon granting and acceptance of the sabbatical leave, you are obligated contractually to fulfill your sabbatical plans as approved by the Committee. Any variation from the approved plan must receive prior approval from the Committee. Payment of salary and benefits is contingent upon this agreement.

Congratulations on the success of your application. I hope that your sabbatical will prove to be of mutual benefit to you and the College.

Sincerely,

Walter W. Collins

Walter W. Collins, Chairperson
Salary and Leaves Committee

WWC:dcd

cc: Salary and Leaves Committee

May 17, 1983

Mr. Walter Collins, Chairman
Salary and Leaves Committee

Dear Mr. Collins.

Regarding modification of my Sabbatical Leave proposal.

My original Sabbatical Leave proposal involved a one semester program of travel and independent study directed toward the development of a Microbiology Laboratory manual relevant to the needs of Mt. San Antonio College students. The proposal also included the writing of two papers, one on the psycho-biological aspects of the tactile senses and the other on reproductive technological developments.

I respectfully request that the Salary and Leaves Committee allow me to modify my Sabbatical Leave proposal to delete the two papers mentioned above.

Rationale for modification.

The primary purpose of the Sabbatical Leave was the preparation of a Microbiology Laboratory manual which would, as a direct result of visitations and study, (1) present those principles and methodologies fundamental to Microbiology and the fields served by this discipline, and (2) provide our students with the most relevant, career-oriented information and laboratory experiences.

While the travel, visitations and studies related to the manual were completed last semester, the work on the manual continues. It has now expanded to include approximately 86 individual experiments. The development and writing of each involves exacting attention to details, many revisions of the manuscripts, artwork, graphs and tables, and has required a far greater expediture of time and effort than was anticipated when the proposal was first submitted.

While the two papers would be interesting and informative, the impact on the instructional program would be limited. The burden of their preparation interferes with the substantial work still required in the preparation of the laboratory manual.

Considering the primary purpose of my Sabbatical Leave proposal, the visitations and study required for the development of the scope of the manual, the time involved in its preparation, the time frame for its completion, and the potential for the greatest benefit to the instructional program of the college because of the many students who will use it, I request that the Microbiology Laboratory manual, the travel and the related visitations and study be accepted as fulfilling the college's requirements for granting Sabbatical Leases.

I thank you for your consideration.

Sincerely,

Betty Ward

Betty Ward

MT. SAN ANTONIO COLLEGE
COMMUNITY COLLEGE DISTRICT
1100 NORTH GRAND AVENUE • WALNUT, CALIFORNIA 91789
Telephone: 714/594-5611

May 19, 1983

Mrs. Betty Ward
Biological Sciences
Campus

Dear Mrs. Ward:

At its meeting of May 18, 1983, the Salary and Leaves Committee approved your request regarding your change in sabbatical leave plans. The Committee was in agreement that the manual you are developing would be a very worthwhile activity. Please be reminded that no future changes in your sabbatical plans should be made.

Best wishes for an enjoyable and educational program.

Sincerely,

Walter W. Collins, Chairperson
Salary and Leaves Committee

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cc Salary and Leaves Committee

CONTENTS

STATEMENT OF PURPOSE

The purpose of my Sabbatical Leave was to enrich lecture and laboratory material, to broaden my knowledge and experiences, and to prepare a Microbiology laboratory manual which would, as a direct result of travel, visitations, and study, present those principles and methodologies fundamental to Microbiology and provide our students with the most relevant, career-oriented information and experiences.

SABBATICAL LEAVE REPORT

My Sabbatical Leave may be divided into three activities: (1) travel within Southern California and Eastern United States consisting of visits to institutions, businesses, medical facilities and government agencies as well as museums, historical sites and points of interest; (2) study and organization of information and materials gained through my travel for the purpose of enriching lectures and laboratory course material and developing relevant Microbiology laboratory exercises, and (3) writing and illustrating a Microbiology laboratory manual for use by Mount San Antonio College students.

I visited the following institutions, medical facilities, government agencies and businesses in Southern California and in the East for the primary purpose of preparing a Microbiology laboratory manual which would, as a direct result of these visitations and subsequent study, present those principles and methodologies fundamental to Microbiology and the fields served by this discipline, and thereby provide our students with the most relevant, career-oriented information and laboratory experiences:

Food and Drug Administration

National Aeronautics and Space Administration (NASA), Virginia

Langley Air Force Base, Langley Research Center, Virginia

California State Health Services:

Food and Drug Section

Investigation Branch

Public Health and Medical Services, Epidemiology Section

University of California at Irvine

University of Southern California

University of Tennessee

University of Virginia

California State University at Fullerton

California State University at Long Beach

University of California at Los Angeles

California State Polytechnic University at Pomona

Loma Linda Medical Centers

University of California at Irvine: Irvine Medical Center

City of Hope Medical Center

Fairview State Hospital (severely handicapped patients)

Medical Laboratories:

- Saint Joseph Hospital
- Saint Jude Hosptial (teaching hospital for medical laboratory technitions)

Long Beach Veterans Administration Hospital

Oakridge Medical Center: Radiation Laboratories, Tennessee

Union Rescue Mission: Medical Facilities

Food Production and Processing Facilities:

- Hunt Foods, Inc.
- Kraft Foods
- Knudsen Dairy
- Alta Dena Dairy
- Brookside Winery

San Gabriel Sewage Treatment Plant

Metropolitan Water District:

La Verne

Orange County Coroner's Office

Cardiovascular Devices, Inc.

American Edwards (artificial heart valves, etc.)

Prior to each visit, I prepared a list of questions I wished to ask and things I wished to observe. I was specifically interested in the type of work or research being done, the application of advanced technologies, the specialized equipment used and the skills involved in their use, the training and/or laboratory experience of the personnel, the microbiological procedures and media used, the types of microorganisms typically used or encountered, and the organization of the laboratories for efficiency and safety. I was also interested in learning of advances in research and technology which might have recently altered the standard procedures, techniques and equipment used in such areas as the laboratory isolation, cultivation, and indentification of microorganisms, the collection, transport, storage and identification of clinical microbial specimens, the investigations of contamination of foods and water, the control of microorganisms, and the testing of antibiotics, antiseptics and disinfectants.

I also asked questions concerning the relevancy of the microbiological training, specifically the laboratory experiences, for the various careers. These included such questions as:

- Are most individuals appropriately trained when entering work in this field? If some training must be done, is this because of the uniqueness of the work being done in the laboratory?

- Does the first semester Microbiology laboratory course adequately prepare the student for advanced microbial studies? If not, how should this be remedied?
- Are there concepts, skills and procedures that should be emphasized more or need to be added to the Microbiology curriculum?

As I visited the colleges and universities, I noted the facilities, the organization of the laboratories and laboratory preparation areas, the equipment, and the safety precautions taken. I obtained copies of course outlines, lists of texts in current use and the microorganisms and media used in laboratory exercises. Previous to my Sabbatical Leave, I had contacted or visited community colleges in Southern California gathering information on Microbiology laboratory course material, equipment, and facilities.

From the information collected through visitations and current texts and laboratory manuals, those areas requiring greater emphasis or additional studies were defined. The following is a summary of the concepts, skills, and laboratory experiences my studies indicate are important for first semester Microbiology students in addition to the basic Microbiology laboratory curriculum. For convenience in reading, I have included with each topic a discussion of the experiments in the laboratory manual (copy attached) that were developed in response to these concerns.

- Require strict and consistent adherence to aseptic techniques.

All facilities visited emphasized the importance of aseptic techniques. Since the Microbiology laboratory is a place where microorganisms are cultivated. tested and stored, accidental contamination of the cultures or personnel can be a serious problem. While the microorganisms used in the

first semester Microbiology laboratory, and in this laboratory manual, are considered non-pathogenic, the student must be made aware that, though the chance is remote, any microorganism given the proper circumstances may become a potential pathogen or be contaminated with a pathogen. Certainly, in the clinical laboratory, as well as in other fields, pathogens and contamination are a very real problem, indeed, threat.

In Experiment 1, I have emphasized the presence of microorganisms on, in and about us. This knowledge is of particular significance to the microbiologist who must consistently use precise techniques to prevent contamination of pure cultures from environmental sources. Experiment 2 demonstrates to the student that there is a direct relationship between aseptic technique and pure cultures, successful experimental results and the safety of laboratory personnel. It provides the initial experiences in the proper techniques using a variety of methods to transfer living cultures from one media to another. Experiment 3 is an extended test of the students ability to maintain sterile tubed media through repeated simulated transfers of microorganisms. Experiment 4 further emphasizes the importance of aseptic techniques through more advanced conditions by requiring the student to maintain a pure stock culture for fifteen weeks. During this period working cultures for other laboratory experiments must be made. Any failure to maintain pure cultures will affect all test results and can only be corrected by considerable effort. I feel these experiments appropriately emphasize the absolute necessity for the mastery of this skill.

- Require strict adherence to safety precautions involving the culturing, and testing of microorganisms and the disposal of contaminated materials and toxic wastes.

All facilities visited strongly emphasized their concern on this matter with the exception of certain academic institutions where facilities were not adequate or equipment was not available for the proper disposal of potentially hazardous materials.

I have required, and so stated in the laboratory manual, that the student must recognize the safety precautions and accept laboratory regulations and safety procedures as prerequisites for work in the laboratory. Information on the accepted means of disposal of contaminated or toxic materials has been included and supplies have been made available for use in the event that live cultures are spilled, ie. Bio-hazard bags.

- Require the preparation and maintenance of accurate, concise experimental records using appropriate terms and format.

Research laboratories such as those at NASA and Langley Research Center and governmental laboratories such as the Food and Drug Administration and the California State Health Services as well as the laboratories at the Metropolitan Water District facilities stressed this concern indicating the necessity for language skills, knowledge of proper terms and the use of appropriate formats. While medical laboratories did not mention this requirement, it was apparent that this was a routine practice in the clinical laboratory.

This requirement has been filled by requiring that students keep a log book in which must be written all experiments performed during the semester. It must be kept up-to-date and present all information in a brief, organized and easily understood manner, using the scientific method format.

- Introduce the concepts of standardized test methods.

The standardized test methods provide credibility to data in that the

results obtained by all laboratories using these procedures may be expected to be comparable. These procedures were developed through experimentation in order to provide the most effective means of obtaining consistently reliable data. Experiments were developed using standard methods for the evaluation of quality control procedures, the levels of contamination in water and in foods and in the evaluation of antibiotics, antiseptics and disinfectants. Experiment 48 is an appropriate example of standardized tests as they apply to the determination of the degree of sensitivity of bacteria to various antibiotics. These procedures provide reliable information concerning the appropriate choice of antibiotic and the most effective concentration to use against specific pathogens isolated from clinical specimens. It offers the physician exact information for planning the antibiotic therapy required by the patient. Strict adherence to standardized procedures by the student will also help develop an attitude of preciseness important in any laboratory endeavor.

- Require the identification of unknown microorganisms under precise conditions including the efficient and knowledgeable use of media.

This concern was of importance to water and sewage treatment plants, epidemiological laboratories, clinical laboratories and the Food and Drug Administration. The Metropolitan Water District, the San Gabriel Sewage Treatment Plant and the Food and Drug Administration require their microbiologists to identify unknown bacteria yearly in order to maintain certification.

To attain such skill, the individual must be able to perform series of microscopic and metabolic tests of the microorganisms. This involves considerable knowledge of the comparative metabolic activities of microorganisms

and of staining procedures. The laboratory manual has been designed to provide the student with an opportunity to develop these skills and to develop flow-charts for the identification of a variety of known bacteria before they are required to identify unknown bacteria. The presentation of the material in this manner provides information as to how classification keys are developed and how to use such keys. I am particularly proud of this innovation.

- Provide opportunities to use a wide variety of test media, to gain information as to their function and usefulness and the reaction of various bacteria when grown on them.

This particular concern was stated by all clinical laboratories.

I have included a major section on enrichment, selective and differential media. One experiment investigates fourteen different media routinely used for primary isolation. Most clinical specimens contain a mixed flora of microorganisms. Primary isolation media is used to determine the predominating species, to differentiate species and to selectively encourage the growth of suspected pathogens. Knowledge of these important media is necessary for work within a clinical laboratory and in the health professions.

Though none of the laboratories mentioned the environmental factors that function as selective agents in the isolation of specific bacteria, all contain variable temperature aerobic and anaerobic incubators which were in routine use. Studies of these environmental factors, as well as others, was included in the laboratory manual.

- Require knowledge of the metabolic pathways of microorganisms and the related natural cycles of materials.

NASA microbiologists and laboratories doing environmental studies were most interested in the inclusion of this material.

This requirement has been met by a series of experiments which investigate the metabolic activities of a wide variety of microorganisms. These include the hydrolysis of polysaccharides, proteins and lipids, the determination of carbohydrate fermentations and the specific end-products produced, the determination of nitrate reduction, urease, oxidase and catalase production, hydrogen sulfide production and citrate utilization. While certain of these studies are included in most laboratory manuals, the relationships of metabolic pathways to the natural cycles of materials has been added to the lecture and laboratory material. These studies are of great value to those entering the environmental and biological sciences.

- Include information on the major disease causing organisms.

This concern was emphasized by the Public Health and Medical Services, Epidemiology Section and the California State Health Services as well as by medical technicians and nurses.

This concern was met by the inclusion of a study, developed by Ed Sounheim of Mt. San Antonio College, that includes thirty-six common pathogens. It requires that students research the staining reactions, the method of transmission, the incubation period, the tissues affected, the disease symptoms, the diagnotic laboratory procedures and the means of prevention of each of the pathogens. A practicuum is given to test this knowledge.

- Provide an opportunity to become familiar with the equipment most frequently used in laboratories including membrane filter apparatus, spectrophotometers, electrophoresis and serological equipment, water baths, shakers, centrifuges and scales.

This concern was expressed by laboratories that have hired individuals with one semester Microbiology laboratory experience who were unable to use

the most basic laboratory experiment.

Experiments have been included that require skill in the use of the membrane filter apparatus to determine the levels of contamination in food and water samples. Also included is a study requiring the use of serological equipment for the identification of microorganisms and in the detection and identification of blood stains and adulterants in meat products. Such experience and skill is important in many laboratories.

Other experiments using a wider variety of equipment will be added as the equipment and materials become available to our laboratory. I respectfully suggest that monies be set aside to modernize the microbiology laboratory and preparation room so as to provide a safer, more practical, efficient and well-equipped teaching laboratory.

- Provide an opportunity to perform more advanced research techniques.

This suggestion was made by many of the areas visited. They felt that students in basic laboratory courses needed the challenge and experience of more advanced and complex experiments.

Many advanced research investigations have applications at the first semester Microbiology course level and provide an opportunity to use the newly acquired skills. I have included a medical-legal serological study which requires the manipulation of minute quantities of materials, ie. antigens, antisera and various reagents. It involves a greater complexity of experimental format than previously encountered. In the study, students are expected to determine whether a stain on a piece of cloth is blood, human blood, theirs or another student's, or chicken, equine or bovine.

Other studies, typical of those performed by Health Departments, require the determination of the effectiveness of sanitation procedures on eating utensils, cups, etc. Another tests the microbial load in foods from vending machines, restaurants, TV dinners, and home refrigerators. These studies require the use of standardized procedures for the collection, transport, and testing of samples and therefore reinforce the student's experiences in this area.

The development, writing and illustration of the laboratory manual has required a period of more than seven months exclusive of the time involved in travel, visitations, and study. Considerable study and organization was required to develop a manual which would provide our students with the relevant concepts, methodologies, and technical skills basic to their educational needs, interests, and future careers. Each concept had to be investigated in order to determine the most effective procedure, microorganisms, media, and incubation conditions so as to produce a viable experiment. Considerations had to be made to control the costs of the experiments, the time frame involved in the execution of the experiments and the efficient use of materials and equipment available in our laboratories. The writing of this manual has proved to be a very valuable and rewarding experience.

My travel in the East not only provided an opportunity to visit more distant institutions and government agencies that might provide unique experiences and pertinent information, but also provided an opportunity to tour museums, historical sites and relevant points of interest. This was a particularly informative part of my Sabbatical and functioned to enhance lecture and laboratory material and bring first hand experiences to the classroom.

CONCLUSIONS

Through travel, visitations and study, I have completed the goals proposed for my one semester Sabbatical Leave: (1) I have enriched lecture and laboratory material, (2) I have increased my knowledge and broadened my experiences, (3) I have defined the concepts, methodologies, skills and experiences applicable to a first semester Microbiology laboratory course and the diverse career goals of our students, and (4) I have written a Microbiology laboratory manual to meet these needs.

I sincerely appreciate this opportunity for travel and study and to write the laboratory manual. It is my purpose and desire that the knowledge and experiences gained and the laboratory manual will serve our students well.

Take interest, I implore you, in those sacred dwellings which one designates by the expressive term: laboratories. Demand that they be multiplied, that they be adorned. These are the temples of the future—temples of well-being and of happiness. There it is that humanity grows greater, stronger, better.

Louis Pasteur

UNIT I

CONTENTS

GENERAL LABORATORY INSTRUCTIONS

The primary purposes of the Microbiology Laboratory are to demonstrate the principles and methodologies of Microbiology and to provide, through experience, the opportunity to develop consistently reliable skills and techniques. The laboratory manual is a guide to introduce the concepts and techniques of microbiological investigations. It leaves you to consider the many variables that present themselves when working with living organisms in the laboratory environment. It is a challenge and a necessary skill to determine why some results differ from the expected. Through such intellectual curiosity, you will learn much more than the original concept on which the investigation was based. You will gain that attitude of analytical perserverance so essential in medical and research sciences. Some procedures may have to be repeated many times before mastery is attained. Do not be satisfied with unsuccessful results due to poor preparation or careless work.

It is essential to study and plan each laboratory investigation before class. Since you will be performing several experiments during the same laboratory period (preparing materials for one investigation, inoculating for another, and collecting the results from still another), it is important to schedule your laboratory work efficiently.

A bound laboratory log book will be kept in which you will record all data and observations. All observations, no matter how seemingly insignificant, should be entered. A useful log book is neat and presents information in a brief, organized, and easily understood manner. Keep your lab book up-to-date, as periodic, unannounced checks will be made.

- No unauthorized visitors will be allowed in the laboratory.
- You will be assigned a microscope, a laboratory storage cabinet and a well-equipped work area. Other equipment and materials will be provided as required. It is your responsibility to properly care for you equipment and experimental materials. Report damaged or missing items to your instructor.
- Some supplies and equipment are shared with other members of the class. Return them to their proper place promptly.
- Never remove equipment, media, or microbial cultures from the laboratory.
- All cultures must be properly labeled with your name or initials, the name of the organism or test procedure, and the date. *They must be discarded promptly at the completion of the experiment.*
- *All contaminated glassware and materials are to be placed in the container labeled* **CONTAMINATED**. These will be autoclaved. *Never discard contaminated items in the wastebasket.*
- *Contaminated pipets are to be placed in the cannister labeled* **CONTAMINATED**. It contains a bacteriocide.
- Flame sterilize inoculating loops and needles thoroughly before setting them down.

- All slides must be cleaned after use.

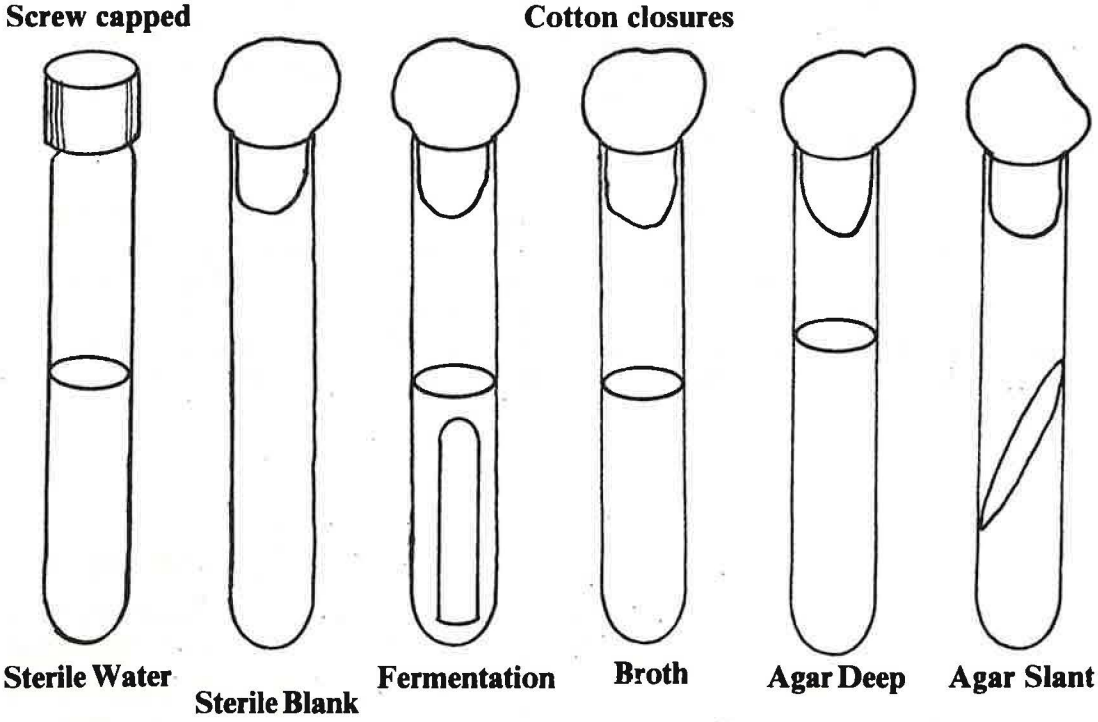
  Stained slides: Organisms on stained slides have been killed by the heat-fixation and the stains. They may be washed without further decontamination.

  Live mounts: Organisms mounted on slides in living condition, such as wet mounts, hanging drops, and negative stain slides, must be killed by placing them in bacteriocide before washing.

  Prepared slides: Remove the immersion oil from prepared slides before returning them to the slide trays.

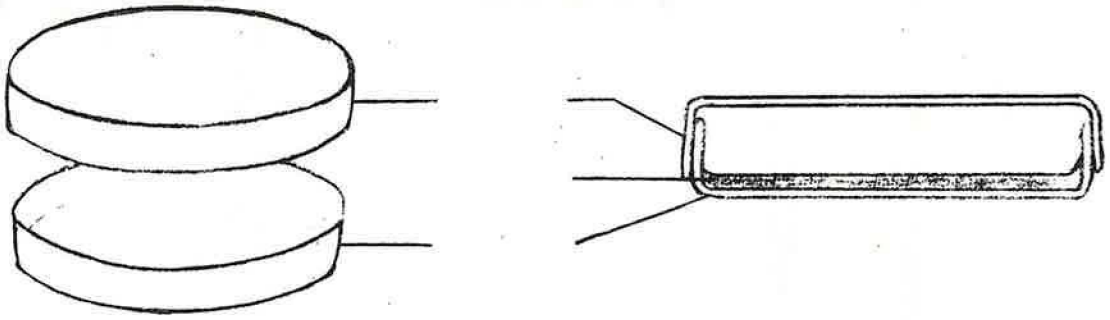
- In the event of fire or personal injury, report the emergency to your instructor immediately. Know the location of the nearest fire extinguishers and how to use them.

- In the event live cultures are spilled: Pour disinfectant on all broken glass and contaminated surfaces. Extend coverage to about 3" around the contaminated area. Cover spill area with paper towels and wait **30 minutes**. Wearing rubber gloves, pick up all glass, residue, and paper towels and place in a Bio-hazard bag. Disinfect area again and wipe dry. Seal the plastic bag and place in container labeled **CONTAMINATED**. Wash hands thoroughly.



Test Tubes

Test tubes are used to cultivate and examine the biochemical and physical characteristics of microorganisms within a sterile environment. The sterile conditions within the tubes are maintained by cotton plugs, plastic or metal caps, or plastic screw caps.



Petri Plates

Petri plates consist of two dish-like parts, one of which fits over the other forming a lid. The disposable Petri plates obtained commercially are presterilized. The standard plate requires about 15 ml. of solid media, the amount contained in one agar deep. The agar deep is melted and then aseptically poured into the sterile Petri plate. The lid is replaced and the agar allowed to solidify without being disturbed.

When incubated, the inoculated plates are inverted to prevent the agar from drying out too rapidly. Agar plates provide a greater surface area than agar slants. They are used to isolate microorganisms in the form of colonies, to test bacterial sensitivity to antibiotics and specific chemicals, and to count the number of microorganisms contaminating foods and water, for example.

Inoculating loops and needles are instruments made of a wire of an inert metal, such as nichrome or platinum, attached to a metal shaft that serves as a handle. The wire may be either straight, a *needle*, or looped at one end, a *loop*. Each serves a distinct purpose.

- Loops are made in several sizes and effectively deliver specific amounts of liquid media. Therse may be used in loop dilution studies to enumerate bacteria. They are also commonly used to transfer cultures from both broth and solid media.
- Needles are used to pick up isolated cultures from agar plates for transfer to pure culture media and to inoculate agar deeps by stabbing.

Swabs

The swab, the most useful piece of collecting equipment, is made of a wooden applicator stick tipped with Dacron batting or long-fibred, medicinal cotton. Commercially prepared swabs are typically buffered, dried, packaged, and gas sterilized. Some are packaged in test tubes to permit sterile transport to the laboratory. The swab, with material from the patient or study site, is used for inoculation to appropriate media.

INCUBATION

Incubators and Water Baths

An incubator is similar to an oven that is thermostatically controlled. For most purposes, it serves the need for incubation at elevated temperatures. Sometimes a more rapid transfer of heat is required and a water bath is employed.

The cultivation of microorganisms requires that they be grwon at their optimum temperature. Most microorganisms pathogenic to humans grow best at temperatures that approximate that of the human body, 35—37° C, while others do not do well at elevated temperatures and should be incubated between 20—25° C. Aerobic and facultative anaerobic microorganisms usually complete a growth cycle within 18 hours at 37° C while strict anaerobes take approximately 48 hours. Organisms incubated at room temperature will grow in approximately 48 hours.

In the microbiology laboratory, it is mandatory that all media and equipment used to culture microorganisms be sterile, free from all forms of microbial life. Three methods of sterilization are commonly used in laboratories.

Dry Heat

Dry sterilization is achieved in a standard household or laboratory oven and requires that high temperatures be maintained for long periods of time. It is most useful for the sterilization of glassware and other inert objects. A typical sterilization cycle is 160° to 180° C for 1½ to 3 hours.

Moist Heat

The autoclave involves the use of steam under pressure, much like a pressure cooker. It is useful for most types of media, cloth, rubber, and other materials that would be destroyed by dry heat. The standard sterilization cycle is 121° C for 15 minutes at sea level. The length of the cycle will vary somewhat with the amount and type of material being autoclaved.

Filtration

Bacterial filters remove bacteria by the sieve-like action of the minute pores of the filter. They are used to sterilize heat-labile solutions that are destroyed or decomposed by high temperatures and to determine bacterial concentrations in fluids.

The most commonly used filters are the membrane filter and the Seitz filter. The membrane filter apparatus uses a cellulose membrane with a pore size sufficiently small to trap and remove bacteria. The Seitz filter functions on the same principle but uses an asbestos filter disc. Each of these filters are used by attaching them to a suction flask.

1

Take a pinch of soil. Hold it between your thumb and forefinger. You may be holding as many as 200 million bacteria.

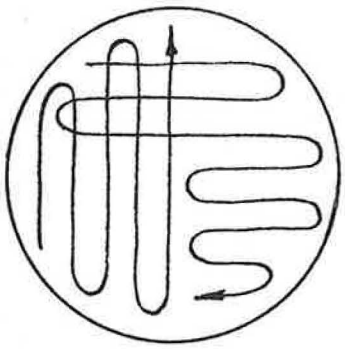
ENVIRONMENTAL MICROORGANISMS

Though the number and kinds of microorganisms differ from place to place, depending on environmental conditions, they are abundant and in or on everything. They are numerous in the soil, in the waters and sediments of oceans, lakes, and streams, and on dust particles carried by the air currents. The few exceptions are in such areas as volcanoes where temperatures exceed the tolerances of even the most specialized microbe, and in healthy, living tissue where the body's natural defenses provide considerable protection from infection. Large numbers of microorganisms are always present on the skin, mucous membranes, and in the intestinal tract of humans and animals. Individuals whose health has been compromised, especially patients in hospitals where harmful bacterial species become indigenous to the hospital environment, are most susceptible to infections. Fortunately, most microorganisms are not harmful; in fact, many are beneficial. The laboratory, like all other environments, is populated with many microorganisms. This is of particular significance to the microbiologist who must consistently use aseptic techniques to prevent contamination of material from environmental sources.

Because microorganisms are invisible, their presence in any environment must be demonstrated experimentally. This is accomplished by exposing nutrient media to the air or by inoculating the media with material from other sources and observing their growth. The types of microbial colonies that develop may be differentiated from one another by size, shape, color, and consistency. Such characteristics are the result of the environmentally-influenced genetic determinants of each organism. Each well-isolated colony consists of many microorganisms that are the progeny of a single cell. Therefore, each colony represents one microorganism isolated from the environment source and is counted as such. This experiment will demonstrate the presence, number, and types of microorganisms from different environments and the absolute necessity of aseptic techniques within the Microbiology Laboratory.

The procedures to be followed in this experiment, and in subsequent experiments, are divided into two parts. The cultures will be set up and incubated during the first laboratory period. You will make your observations and record your results during the following laboratory period.

4. Inoculate the sterile agar plates in the manner illustrated. The air, hair, and clothing samples have been inoculated by the open exposure technique and **should not be streaked**.



Cotton swab isolation. The swab streak procedure may be used to isolate individual colonies after incubation. At least one-quarter of the surface will demonstrate isolation if the inoculum was not too concentrated.

5. Invert all plates and incubate at the designated temperature until the next laboratory session.
6. Log the inoculations.
7. Discard all contaminated materials and equipment in the designated containers.

Observations

1. Examine the 21° C and 37° C incubated plates.
2. Count the number of colonies on the surface of the plates exposed to air, hair, and clothing.
3. Count the number of colonies in the quarter of the swab-streaked plates that has well-isolated bacterial colonies rather than confluent growth and multiply by four. This will not give an accurate count, but if all streaked plates are counted in the same manner, an estimation of differences in numbers between the various sources can be made.
4. Determine the number of different colony types based on colony size, shape, elevation, color, and texture. These are best evaluated when individual colonies are well isolated from others.
5. Examine the plates of other teams.
   a. Compare the differences in numbers and types of colonies from the same sources but incubated at different temperatures.
   b. Compare throat cultures, noting similarities and differences.
   c. Examine plates from at least two habitats noting the differences in colony types.
6. Record your results and observations. The following are examples of discussion topics:

   — What types of environments seem to have the most numbers? The least numbers? Can you give a possible explanation for this?

   — Would you say that most of the bacteria grown in this experiment are disease-producing, considering the source and incubation temperatures?

   — How does this experiment relate to sterile technique and prevention of contamination?

2

ASEPTIC TECHNIQUES

Since microorganisms are everywhere and you are working with a high density of organsims, certain procedures, called **aseptic techniques**, must be followed to prevent contamination during the handling of laboratory cultures. *There is a direct relationship between aseptic technique and pure cultures, successful test results, and the safety of laboratory personnel.* In this experiment, you will aseptically transfer pure cultures of bacteria to liquid, semi-solid, and solid media. The process of transferring cultures from one medium to another is called **subculturing**. The abilty to subculture safely, without contamination, is one of the most essential techniques of the microbiologist. Though the procedure is simple, you will probably be uncomfortable and unsure of your technique. With practice it will become routine and automatic.

General Information about transferring

- The inoculating loop or needle is generally made of 24 or 26 gauge nichrome wire. Under certain circumstances, such as in serological tests, nichrome wire is unsuitable because of its iron content. Platinum wire should be used.
- Generally, an inoculating needle is used to transfer microorganisms from a solid medium and an inoculating loop is used to transfer microorganisms from a liquid medium.
- The inoculating loop or needle must be steilized immediately before and after use. Flame the entire wire to incandescence by first heating the end of the wir nearest the handle. Gradually move the wire through the flame until all parts have been sterilized. Then quickly flame several inches of the handle adjacent to the wire. To avoid spattering or the formation of hazardous microbial aerosols, wet or contaminated wires should be dried over the flame and then flamed as usual.
- Never touch a hot wire to a bacterial culture or specimen or rattle the wire in a tube; a contaminating aerosol will result.
- Once the inoculating instrument is sterilized for use, do not set it on any surface or allow it to touch anything.
- During transfers using tubed materials, remove and hold the caps or cotton plugs between the fingers of the right hand, if right-handed. Never set them down. If one is dropped, replace it immediately with one from a sterile blank.
- Flame sterilize the openings of culture and media tubes bedore and after transferring microorganisms.
- During inoculations, tube and plate cultures and media must be held in such a manner as to protect them from air-borne contaminates. Tubes are to be held horizontally or nearly so, and plates held vertically or only partially opened.
- When transferring with sterile pipets, do not uncover them until ready to use them. Never touch the tip or allow it to touch anything. After use, return it to its cover and discard.
- Record all inocultions in your log book.

B. Loop transfer of a broth culture to an agar plate

1. Repeat procedures 5 through 10 given for the loop transfer of a slant culture to an agar plate using a broth culture. **NOTE:** Shake the broth culture to suspend the cells within the media. Do not allow the cotton plug to get wet.
2. Inoculate the surface of the agar labeled *broth*.
3. Sterilize the loop and set it on the rack.
4. Incubate the inoculated agar plate at 21° C until the next laboratory session. Petri plates must be inverted for incubation to avoid disruption of colonies by condensation droplets.

C. Loop transfer of a broth culture to an agar slant

1. Flame the inoculating loop and allow it to cool.
2. Hold the broth culture and agar slant tubes between the fingers of the left hand.
3. Remove the cotton plugs with the fingers of the right hand and insert the loop into the culture broth.
4. Transfer the loop to the agar slant and place the loop at the bottom of the slant and gently draw it up making a wavy line. Do not scratch the surface.
5. Flame the mouths of the tubes and replace the cotton plugs. Set them in the rack.
6. Flame the inoculating loop and place it on the rack.
7. Incubate at 21° C until the next laboratory session.

D. Pipet transfer of a broth culture to a broth

1. Open the package containing the sterile pipet by tearing off the end nearest the mouth of the pipet.
2. Hold the pipet in the right hand with the index finger over the mouth. **NOTE:** *The index finger controls the rate of flow of the liquid from the pipet.*
3. Hold the broth culture tube and broth tube in the left hand and remove the cotton plugs between the fingers of the right hand.
4. Flame the mouths of the two tubes.
5. Immerse the tip of the pipet into the fluid in the broth culture tube and place your **dry lips** around the mouth end of the pipet and slowly draw the fluid into the pipet by creating a gentle suction.
6. With the fluid drawn **above** the zero line on the pipet, quickly remove your mouth and put the tip of the index finger over the hole in the mouth end of the pipet.
7. Relax the index finger just enough to let the fluid drain down to the 0.5 ml graduation.
8. Transfer the pipet to the broth tube and dispense the media. These pipets are blow-out pipets and require that the last drops be blown out to get the proper, measured amount.
9. Flame the mouths of the two tubes, replace the cotton plugs, and set in the rack.
10. Discard the pipet in the appropriate container.
11. Incubate at 21° C until the next laboratory session.

3

ASEPTIC TRANSFER SKILLS

The success of laboratory experiments and the safety of all concerned is dependent on consistent and effective aseptic techniques. This experiment will test your ability to aseptically transfer media from one tube to another, without contamination, for an extended period.

Materials

— **2 tubes of nutrient broth**

Methods

a. Label both tubes with your name, the number of the experiment, and the date. Use a permanent marking pen.

2. Transfer one loopful of the medium from one tube to the other using aseptic technique.

3. Place a rubber band around your tubes and set them in the designated basket on the front desk.

4. Each day as you enter the laboratory pick up your test tubes. The transfers will be made at the first of each laboratory session.

5. Return your tubes to the basket. They will be incubated until the next laboratory session.

6. Continue this procedure until notified in approximately three weeks.

4

MAINTAINING PURE STOCK CULTURES

In the search for the causative agents of diseases for medical treatment or where the etiology and epidemiology are not known, clinical microbiologists must find and isolate the suspect species from patients or the environment and maintain the microorganism in pure culture. Other microbiologists do not customarily use this search and seizure technique but instead order pure cultures from commercial laboratories that maintain cultures of a wide variety of bacterial species as well as specialized strains of these species.

The maintenance of pure cultures and the subculture of these cultures for laboratory studies has not been a part of the Microbiology student's course work. However, it is a necessary skill and is now included.

Materials

- **Assigned bacterial culture**
- **Recommended transfer and growth medium**
- **Media for the preparation of laboratory experimental subcultues**

Method

1. The stock culture of the bacterium must be maintained in pure culture.
2. The bacterium must be transferred regularly to avoid loss of the culture. As the culture ages, nutrients are depleted, metabolic wastes accumulate, and the medium dries.
3. Not all bacteria grow well on nutrient agar slants. Detemine and use the appropriate support media.
4. Use your bacterium for each laboratory experiment and record the results carefully. This information will be used, with the information from others, to form an identification key for the **Unknown Cultures** which will be assigned later.
5. Report any problems to your instructor immediately.
6. Prepare cultures of your bacterium:
   - One **Reserve Stock Culture** will not be used except in case of loss or contamination of the **Stock Culture**. This culture must be kept in the refrigerator after being established.
   - One **Stock Culture** will be kept in your storage area and used for preparing **Working Cultures.**
   - One **Working Culture** will be subcultured when required. Check the laboratory schedule and prepare a working culture 24 hours before needed. Some experiments require older cultures. Ask if you are not sure!

UNIT 2

CONTENTS

THE COMPOUND LIGHT MICROSCOPE

"Where the telescope ends, the microscope begins;
Which of the two has the grander view?"

Victor Hugo
Saint Denis, Book III, Chapter 5

The purpose of the microscope (Greek: micros— small; and scopein— to see) is to magnify otherwise unobservable objects. As such, it is one of the most useful tools of the microbiologist. Virtually all organisms studied in a microbiology laboratory are smaller than 200 micrometers (um) which is the approximate limit of resolution of the naked eye. Bacteria range in size from as small as 0.2 um to 40 um. While biochemical and serological techniques are being used to a greater extent for the identification of microorganisms, the basic presumptive tests in the identification of microorganisms, require microscopic examination. These studies permit the determination of microbial size, shape, and cell arrangement, capsule and spore formation, motility and staining reactions, among other things.

The student must, therefore, understand the basic principles of brightfield microscopy; be skillful in the operation and use of the microscope, and exercise proper care and maintenance. (A detailed discussion of the compound microscope has been prepared and placed in the Study Skills Center for your review.)

Materials

- Compound microscope with light source
- Prepared slides of:
  - *Paramecium caudatum*
  - Mixed bacterial cell types

A. Care of the microscope

Each student is assigned a microscope and is responsible for its care and proper use. In that you are sharing your microscope with students from other Microbiology Laboratory classes, it is important you make certain your microscope is clean and in proper working condition at the begnning of each laboratory period. If there are any problems, report them to your instructor immediately.

Since the microscope is a delicate and expensive precision instrument, the following regulations and procedures must be observed:

1. Carry the microscope with the arm of the microscope securely held in one hand and the base supported by the other.
2. Place the microscope gently on the desk away from the edge.
3. Clean the microscope before and after use. Use only clean, dry *lens paper* **to remove dust and oil from the ocular and objectives lenses.**
4. **When you have completed your microscope studies:**
   a. **Lower the condenser.**
   b. **Rotate the low-power objective into working position.**
   c. **Lower the body tube completely.**
   d. **Replace the dust cover.**
   e. **Return the instrument to its designated space in the microscope cabinet.**
5. **The microscope cabinet is locked whenever an instructor is not conducting a class. If you wish to use your microscope in the laboratory when classes are not in session, you must sign a microscope check-out slip. You are responsible for the microscope until it is returned to the locked cabinet and your check-out slip has been returned.**

Table 1: The Parts of the Microscope

Microscope Part	Function
Ocular or eyepiece	A series of lenses that enlarge the image 10 times (10x).
Ocular thumb wheel	Controls the distance between the oculars so adjustments can be made to fit your eyespan.
Body tube	A long tube which provides the distance for proper focusing of the image.
Revolving nosepiece	A revolving extension of the body tube which carries the objective lenses and can be rotated to change from one objective lens to another.
Objectives	Three (3) cylinders on the nosepiece which contain several lens systems to magnify the specimen: low-power objective (10x); high dry power objective (43—45x); oil immersion objective (97—100x).
Slide holder	Two (2) metal clips, parallel to one another, which hold the slide in position in the mechanical stage.
Mechanical stage	A graduated, moveable device on the stage which is controlled by two knobs. These move the slide back and forth and from side to side. A specimen can be located by recording the coordinates on the graduated scale.
Stage	Supports the mechanical stage and microscope slide.
Diaphragm with lever	A camera-type diaphragm or iris which regulates the amount of light entering the microscope.
Substage condenser	A set of lenses which condense the light waves. Controls the light intensity when raised or lowered.
Light source	A built-in source of illumination which directs the light through the microscope.
Arm	A supporting back frame of the microscope. Provides a handle for transporting the microscope.
Base	A platform which supports the entire microscope.
Coarse adjustment knob	A large round knob which, when rotated, raises and lowers the body tube. Provides approximate focusing.
Fine adjustment knob	A smaller knob which, when rotated, slowly raises and lowers the body tube. Provides precise focusing to sharpen the image of the specimen being observed.

14. **Use the fine adjustment knob to bring the bacteria into sharp focus. If focus cannot be found after a few moments, return to low power objective and relocate the bacteria. Return to oil immersion objective and focus using only the fine adjustment knob.**

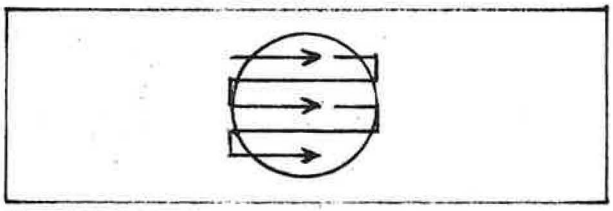
When you have become more adept at working with the microscope, you can use the oil immersion lens immediately without going through the preliminary steps. The procedure is as follows:

a. **Adjust the movable body tube so the objective is as far from the stage as possible.**

b. **Rotate the fine adjustment knob as far as it can be rotated upward.**

c. **Place a drop of immersion oil on the stained specimen.**

d. **Looking at the microscope from the side, rack down the oil immersion lens with the coarse adjustment until it touches the oil and continue downward until the objective is just above the level of the slide. Now look through the eyepiece and slowly rotate the fine adjustment away from the specimen. If you have to rotate the fine adjustment more than five or six turns and cannot get the specimen in focus, repeat the procedure, getting the lens as close to the specimen as possible. As long as you first rotate the fine admustment clockwise, you will not drive the objective lens into the slide during the focusing. This procedure requires a certain amount of technique and experience.**



15. **Scan the slide by examining while moving the slide across the top to the side, then down slightly and across again in a systematic search pattern as indicated:**

Slide Scan Pattern

No additional oil is required for this maneuver. Locate the different bacterial cell types. A slight clockwise then counter-clockwise rotation of the fine adjustment knob will give a three dimensional effect to the image.

16. Draw representative cell types in your log book and indicate the magnification used. Label the cells according to their morphology: Spherical (coccus; plural, cocci), rod (bacillum; plural, bacilli), and spiral (spirillum; plural spirilla).

17. When you have completed the study, clean the slides and return them to the appropriate slide tray.

18. Thoroughly clean the objective lenses with lens paper.

CALIBRATION OF THE MICROSCOPE

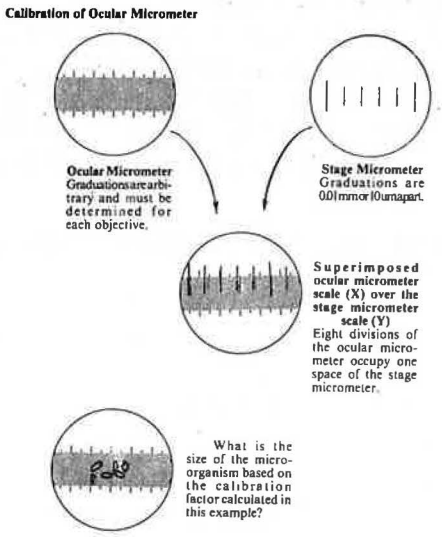
The exact measurement of a microorganism is an important aspect of its description. Before such measurements can be made, the microscope must be calibrated. This process involves the determination of the extent of magnification accomplished by each of the objective lens systems and requires the use of two optical devices, an **ocular micrometer** and a **stage micrometer**. The ocular micrometer is a glass disk with parallel, equally spaced but unmeasured lines etched on its surface. It is placed within the ocular tube. The stage micrometer is a glass slide on which a microscopic ruler has been etched. The graduations of the ruler are 0.01 mm apart. It is placed on the stage between the mechanical stage holders.

By superimposing the image of the ocular micrometer over that of the stage micrometer, the distance between the lines of the ocular micrometer can be calculated. This distance is called the **calibration factor**. Because the extent of magnification achieved by each objective lens system differs, each must be calibrated separately. In fact, individual microscopes must be calibrated for each differs, however slightly, from another. The calibration factor for one ocular division is calcuated as follows:

$$\textit{One ocular micrometer division in mm} = \frac{\text{Number of stage micrometer divisions x 0.01 mm}}{\text{Number of ocular micrometer divisions}}$$

EXAMPLE: In the illustration, eight ocular micrometer divisions fit exactly within one stage micrometer division. Each stage micrometer division equals 0.01 mm. Using these figures, you can now calculate the distance between the ocular micrometer divisions.

$$\text{One ocular micrometer division (calibration factor)} = \frac{\text{0.01 mm (distance on stage micrometer}}{\text{8 (number of ocular micrometer divisions)}} \times 0.00125 \text{ mm}$$



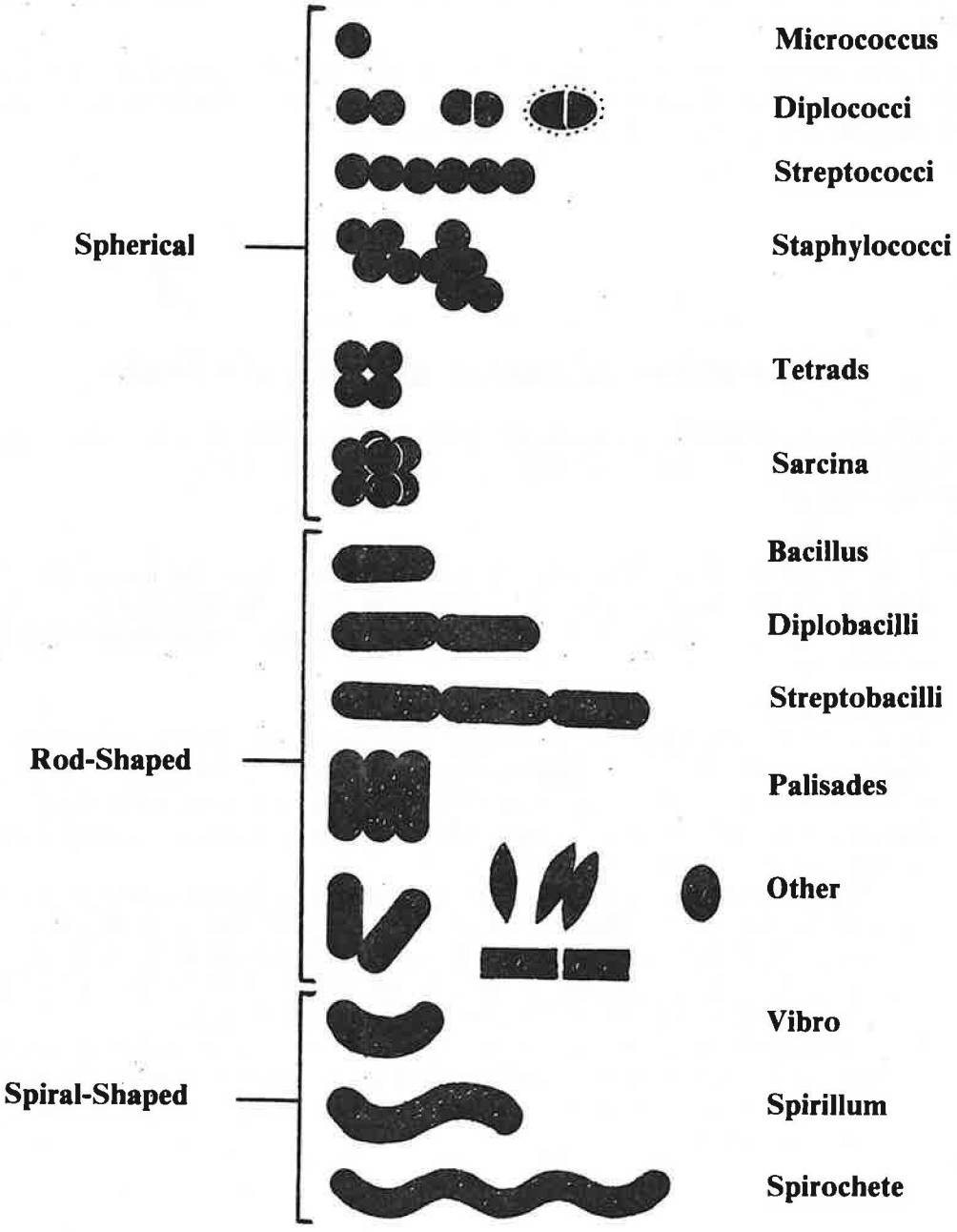
To convert millimeters to micrometers (um), multiply the number of millimeters by 1,000, the number of micrometers in one millmeter. In this example it would be:

0.00124 mm X 1000 = 1.25 um

Bacterial Morphology and Staining Techniques

The size, shape, and cell arrangement of bacteria is typical of the species and useful in differentiation and classification. Since the size of a bacterial cell varies during growth, this characteristic is not appropriate in the identification of similar groups of organisms. However, most bacteria maintain a relatively constant morphology and cell arrangement (with important exceptions) and may be classified on the basis of these characteristics. There are three basic shapes among bacteria: *spherical* (coccus; plural— cocci), *rod-shaped* (bacillus; plural— bacilli), and *spiral-shaped* (vibrio; spirillum; plural— spirilla, and spirochete). Some bacilli are so small they appear as cocci and are called *coccobacilli* and others are *pleomorphic*, exist in different shapes. Bacteria also assume characteristic cell arrangements based on the way cell division and subsequent cell separation occurs in that particular species.

Basic Shapes and Arrangements of Bacterial Cells



Heat Fixation

To **fix** a smear, the slide with the smear on the upper surface is passed three or four times through the flame of the Bunsen burner. This kills the bacteria and causes the bacterial proteins to adhere to the slide. A properly fixed smear will not wash off during the staining procedures and will preserve the structures of the cell in their respective forms.

Simple Stain

In this procedure, the bacterial smear is stained with a single cationic (basic) dye. Since the dye is positively charged, it is attracted to the negatively charged components of the bacterial cell. The purpose of the simple stain is to demonstrate cell morphology and arrangement. Occasionally, endospores may be seen as unstained refractive bodies within bacterial cells.

Materials:

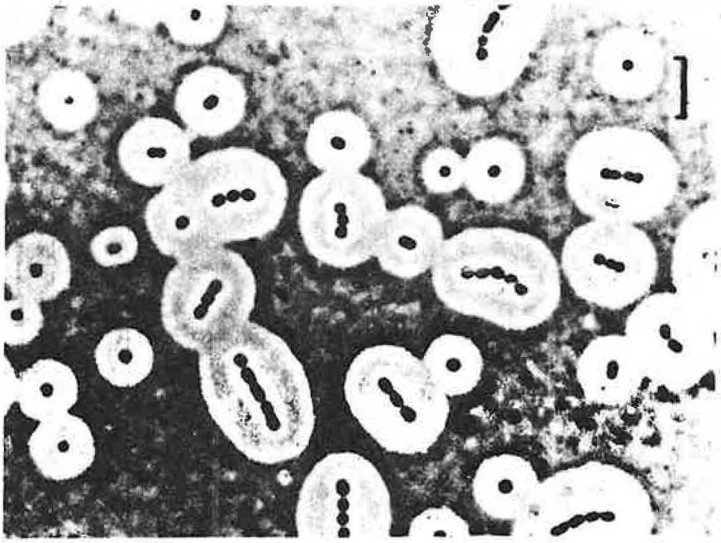
- Slant or broth cultures of:
  Escherichia coli
  Staphylococcus aureus
  Bacillus subtilis
- Methylene blue
- Safranin
- toothpicks

Methods:

1. **Divide each of two slides into two sections.**
2. **Remove a small specimen of material from the gum line of one of your rear molars, emulsify it with water on one of the sections of a slide, and prepare a smear.**
3. **Aspetically transfer an inoculum of each of the three (3) cultures to the other sections.**
4. **Dry thoroughly and heat fix. The drying may be accelerated by holding the slide high above the flame.**
5. **Stain the slide with the oral specimen with methylene blue for 3—5 minutes and gently wash with tap water to remove the excess stain.**
6. **Blot the slide dry between a folded sheet of bibulous paper. Do not rub. The stained smear is now ready for observation.**
7. **Stain the second slide with safranin for thirty (30) seconds and gently wash to remove the excess stain. Blot dry.**
8. **Examine the prepared slides using oil immersion. The stained oral specimen will contain a few epithelial cells. These cells will appear as large flat cells. The nucleus will be stained blue. The bacterial cell will be much smaller and a darker blue. Compare the morphologies of the three bacterial cultures with those in the oral specimen.**
9. **Log your results as drawings and discussion.**

Negative Stain

The negative stain technique requires the use of either India ink which is composed of particles too large to enter the cell or a negatively charged, anionic, stain which is repelled by the normally negative charge on the surface of bacteria. In either method the capsule remains unstained and discernible against the darkened background. If the bacterial cell is treated with a contrasting stain, the clear capsular material is more distinct. Since heat fixation is not required, the cells are not distorted and the natural size and shape may be seen.



Photomicrograph of wet mount of an encapsulated bacterium in India ink. The scale line shows the size of 10 um at this magnification.

The ability of bacteria to produce capsules is an environmentally controlled genetic characteristic. Under normal conditions, most bacteria produce a loosely attached slime layer. Only a few bacterial species produce a well-developed capsule. Cultures of encapsulated species give a stringy texture to fluid media and form moist, glistening colonies on solid media. Capsules tend to increase the virulence of pathogens by inhibiting phagocytosis. In some pathogens the capsules are antigenic and stimulate the production of antibodies which, in the case of Pneumococci, react with the capsule causing a swollen appearance called the Quellung reaction, a clincally useful diagnostic procedure.

Materials

- tyrptose-phosphate slant cultures of:
  - Alcaligenes viscolactis
  - Flavobacterium capsulatum
  - Proteus vulgaris
- India ink (freshly filtered)
- Loeffler's methylene blue
- one (1) small beaker for tap water rinse

Gram's Stain

The Gram's staining procedure is indispensable in the identification of unknown organisms. It is routinely the first test performed in the clinical laboratory and provides the initial clues as to the identification of a pathogen.

The Gram's stain involves the use of four reagents:

1. **Primary stain.** The primary stain, *crystal violet*, functions to color all cells a deep blue.
2. **Mordant.** The mordant, *Gram's iodine*, forms a complex with the dye, fixing it to the bacterial cell.
3. **Decolorizer.** *Acetone-alcohol* or 95 percent alcohol, releases the stain complex from the cell, decolorizing some cells.
4. **Counterstain.** A contrasting stain, *safranin*, replaces the primary stain in those cells unable to retain the primary stain.

When thus stained, bacteria are either blue, Gram-positive, having retained the primary stain, or pink, Gram-negative, having lost the primary stain and been colored by the counterstain. The ability to resist decolorization is related to the chemical make-up and structure of the cell wall. Most cells of living things, including humans, are Gram-negative. It is the Gram-positive characteristic which is distinctive, found only in yeasts, a few molds, and some bacteria. Older cells of some Gram-positive species tend to lose their Gram-positiveness and appear Gram-negative. The culture will then appear **Gram-variable**, some cells Gram-positive and some Gram-negative.

The Gram's staining reaction correlates with important physiological and cytological traits. Gram-positive cells are more sensitive to antibiotics and dye bacteriostasis and require more complex media for growth, while Gram-negative cells are more susceptible to cell digestion by strong alkali and acids.

In such an important technique one should be aware that variations in the Gram procedure critically influence the results obtained. Such factors as the density of the smear, the concentration of the reagents, the length of time used in washing and decolorization must be considered. Excessive decolorizing may cause Gram-positive organisms to lose the primary stain and appear Gram-negative when counterstained, while insufficient decolorizing may allow Gram-negative organisms to retain the primary stain and appear Gram-positive. The reliability of the results is dependent on the skillful use of precise and constant Gram's staining procedures.

Materials

- 24-hour cultures of:
  Staphylococcus epidermidis
  Escherichia coli
- 48-hour cultures of:
  Bacillus subtilis
  Micrococcus luteus
- Gram's crystal violet (primary stain)
- Gram's iodine (mordant)
- acetone-alcohol (decolorizer)
- Gram's safranin (counterstain)

10

Acid-Fast Stain

The Acid-fast stain is a differential stain that compares the resistance of cells to decolorization by acids. Most bacteria are easily stained with carbolfuchsin and, when treated with acid-alcohol, decolorize completely. However, it is relatively difficult to stain certain other microbes with this dye but, once stained, they are just as difficult to decolorize, even with acid-alcohol. Those that retain the carbolfuchsin are called *acid-fast* while all other microorganisms, which are easily decolorized by acid-alcohol, are *non-acid-fast*. This is an excellent differential stain and is used clinically in the identification of the tuberculosis bacillus, *Mycobacterium tuberculosis*, the leprosy bacillus, *Mycobacterium leprae*, and certain species of *Nocardia* which causes nocardiosis, a pulmonary disease. It is also used to differentiate a number of harmless acid-fast saprophytes.

The property of acid-fastness is derived from the presence of many complex lipids, fatty acids, and waxes within the cell wall. Acid-fast bacteria have a high cell wall lipid content, approximately 40 to 60 percent. Gram-negative bacteria have no more than 20 percent and Gram-positive bacteria have only 1 to 4 percent. The lipid content is also responsible for the characteristic rough, wrinkled, dry surface associated with colonies of mycobacteria.

Many textbooks, and Bergey's Manual, state that mycobacteria are Gram-positive. However, it is impossible to Gram's stain an acid-fast organism. First, the stain will not penetrate the cell unless heat or chemicals are used. Second such stained cells will not decolorize with Gram's alcohol. Their retention of the primary stain is due to their acid-fast characteristic rather than to any Gram's staining chracteristic. Therefore, acid-fast bacteria are neither Gram-positive nor Gram-negative.

The acid-fast stain uses three different reagents:

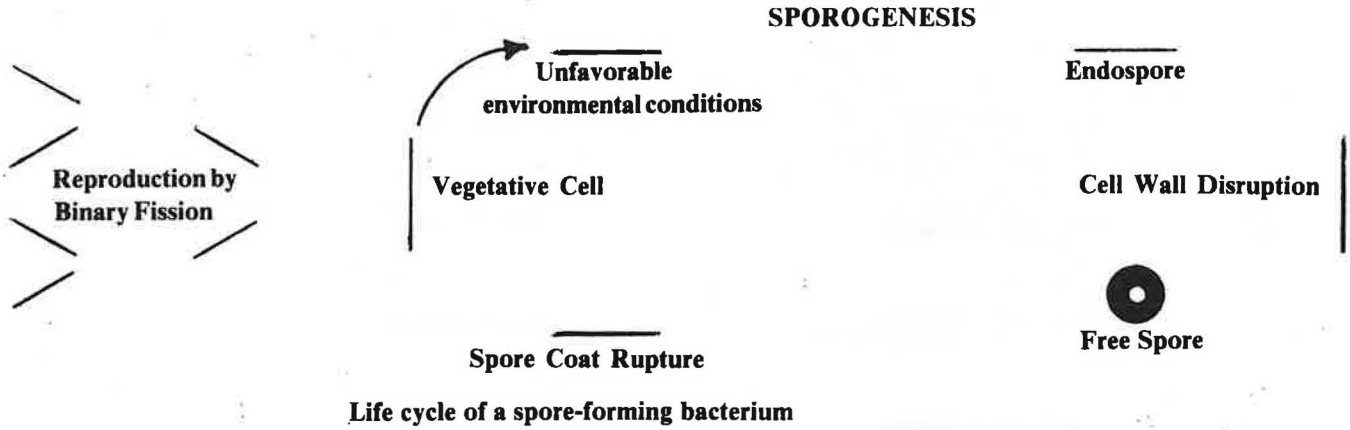
1. **Primary stain.** The primary stain, *carbolfuchsin*, is soluble in lipoidal material and so will penetrate and be retained by the cell wall. Penetration is also facilitated by the application of heat which drives the carbolfuchsin through the wall and into the cytoplasm.
2. **Decolorizer.** Prior to decolorization, the smear is cooled to allow the waxy cell wall substances to harden. *Acid-alcohol* is applied to decolorize the cell. Acid-fast cells resist decolorization and remain **red**. Non-acid-fast cells are decolorized and transparent.
3. **Counter Stain.** *Methylene blue* is used to stain the decolorized non-acid-fast cells **blue**.

Materials:

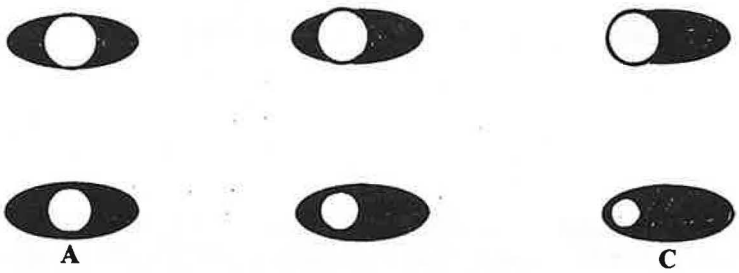
- 48-hour slant culture of *Mycobacterium smegmatis*
- 24-hour culture of *Bacillus subtilis*
- Ziehl-Neelson acid-fast staining reagents
  Carbolfuchsin (primary stain)
  Acid-alcohol (decolorizer)
  Methylene blue (counterstain)

In this study, the Schaeffer-Fulton staining method will be used to differentiate vegetative cells and spores. This procedure involves the use of two (2) reagents.

1. **Primary stain.** The primary stain, malachite green, is steam-heated to facilitate penetration of the impervious spore coats. Both the spore and the vegetative cell are stained green by this process.
2. **Decolorizer.** Once stained, the spore is more resistant to destaining than the vegetative cell, which does not form a strong bond with the primary stain. Water removes the excess stain and leaves the spore a rich green and the vegetative cell transparent.
3. **Counterstain.** Safranin, a red stain, is used to counterstain the vegetative cells a contrasting color.



Life cycle of a spore-forming bacterium



Location of bacterial endospores

Spores may or may not be swollen

Central Subterminal Terminal

Materials:

— 48-hour broth culture of *Bacillus megaterium*
— Schaeffer-Fulton spore staining reagents
  Malachite green (5 percent aqueous solution)
  Safranin (0.5 percent aqueous solution—**not** Gram's safranin)

Method:

1. Prepare a fixed smear.
2. Add malachite green and allow to act on the smear for thirty (30) seconds.
3. Place a small piece of blotting paper over the smear and steam over boiling water for three (3) minutes. Do not allow the dye to evaporate.
4. Wash thoroughly with tap water.
5. Counterstain with safranin for thirty (30) seconds.
6. Wash the slide, blot, and dry.
7. Examine under oil immersion. Are the spores swollen? Terminal, Subterminal, or Central? Log a typical illustration of the spores of this organism.

Wet Mount

An advantage of this method is that the slide is easily prepared and, like the hanging drop technique, it permits viewing microorganisms under natural conditions. It requires that care be taken to distinguish true motility, Brownian movement, and the movement caused by water currents which develop in wet mounts due to heat and gravity.

Materials:

— Young cultures of:
  Bacillus subtilis
  Proteus vulgaris
  Micrococcus luteus
  NOTE: These cultures are also to be used for the hanging drop and motility medium studies
— **Methylene blue (1:5000 dilution)**
— **Cover slips**

Methods:

— **Transfer four loopsful of** *Bacillus subtilis* or *Proteus vulgaris* to a clean microscope slide and add a drop of diluted methylene blue. Place a cover slip over the mixture.
— Place the slide on the microscope stage and focus using the low power objective. Reduce the light intensity.
— Examine the preparation using the oil immersion objective.
— Record your observations. Which of the two methods do you prefer? Would it be helpful to add a loopful of diluted methylene blue to the handing drop slide? Why should young bacterial cultures be used for motility testing?

CAUTION:

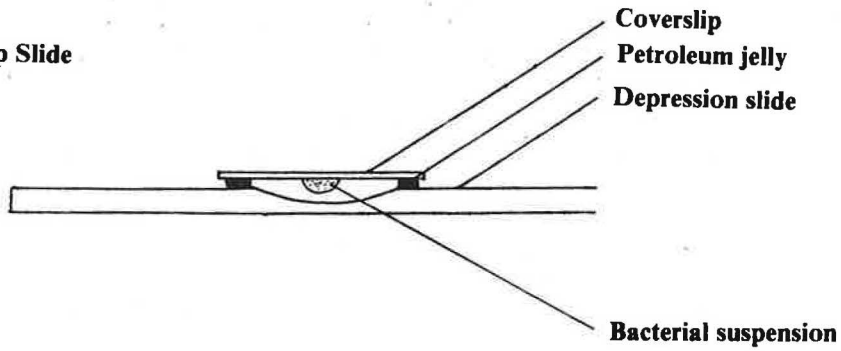
Wet mount and hanging drop slide preparations contain living bacteria. Discard these preparations in the suitable container of disinfectant.

Hanging Drop

In this technique, bacterial motility is observed directly by viewing live bacteria in a liquid medium. Care must be taken to distinguish between true motility and the random motion of brownian movement. An advantage of the hanging drop technique is that it provides an undistorted view of the size, shape, and movement of microorganisms.

Materials:

- Young cultures of:
  Bacillus subtilis
  Proteus vulgaris
  Micrococcus luteus
- Depression slide and cover slips
- Petroleum jelly and applicator sticks



Method:

1. **Thoroughly clean a depression slide. Using an applicator stick, place a thin layer of petroleum jelly around the edge of the concave well.**
2. **Using sterile technique, place a loopful of** *Bacillus subtilis* in the center of the cover slip.
3. Invert the depression slide and place the concave well over the bacterial suspension on the cover slip.
4. Press down gently to form a seal between the slide and the cover slip.
5. Turn the slide over. The prepared hanging drop slide should appear as illustrated with the drop containing the bacteria suspended within the concave depression.
6. Examine the slide first with the low power objective. Greater care in focusing is required because of the thickness of the depression slide. Keep the lighting to a minimum. Unstained bacteria are translucent and difficult to see under bright light.
7. Once the image is seen under low power, rotate the oil immersion objective into position and readjust the lighting. Focus near the edge of the bacterial suspension where most of the organisms are drawn by surface tension.
8. Observe the bacterial activity. Brownian movement and the flow of organisms or particles in currents in the fluid must not be mistaken for true motility, which is a purposeful type of movement.
9. Make a hanging drop slide of *Proteus vulgaris* and compare the difference in movement of the two cultures.
10. Record your observations.

Motility Medium:

Motility tests are generally performed using a motillity medium instead of the hanging drop or wet mount techniques. A motility medium is a semisolid agar in which the concentration of agar is less than that of conventional agar media. Bacteria are inoculated into the medium using the stab technique. The migration of motile bacteria from the inoculation site can be seen after incubation. Motile bacteria move through the semisolid medium and their growth produces turbidity throughout the tube, whereas non-motile bacteria grow only along the line of the stab inoculation.

Materials:

— Young cultures of:
  Bacillus subtilis
  Proteus vulgaris
  Micrococcus luteus
— Motility agar deeps (2)

Methods:

1. Using sterile technique, remove an inoculum of your assigned culture with an inoculating needle and inoculate the motility agar by stabbing down the center of the tube, being careful not to touch the bottom. Withdraw the needle along the stab line so as not to spread the inoculum within the medium.
2. Using a toothpick, collect a specimen of tartar from the gum margins of your rear molars. Transfer the specimen to a sterilized inoculating needle and inoculate the other tube of motility medium using the same technique.
3. Incubate both tubes until the next laboratory period. Examine the tubes and those of your partners for the presence or absence of motility. If growth is restricted to the stab line and is well demarcated, with no growth in the rest of the agar, the organism is considered *nonmotile*. Growth throughout the medium indicates a motile organism.
4. Record all results and conclusions. Do the motility agar studies agree with your observations of the same organisms in the hanging drop and wet mount slides? Which of these three methods do you prefer?

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CYTOLOGICAL CHARACTERISTICS OF AN UNKNOWN BACTERIUM

Bacterial cytology requires the skillful preparation of bacterial slides and use of the microscope. It is the first step in the identification of bacteria. In this study you will be given an opportunity to demonstrate your skill by determining the cytological characteristics of an unknown bacterial culture. You will have one laboratory period to complete your study. Work independently. This is a test of your mastery of these techniques. You may use your laboratory manual.

Materials:

— Unknown bacterium, assigned by number.

Method:

1. Determine the following:
   - cell morphology
   - cell arrangement
   - Gram stain reaction
   - Acid-fast stain reaction
   - motility
   - endospores
   - capsules
2. Log your results and have your log book initialed by your instructor.
3. Write a brief report on your study and turn it in next laboratory period.
4. Indicate which of the following organisms may have been the one assigned to you and state the reasons for your conclusions.
   - *Escherichia coli*
   - *Bacillus subtilis*
   - *Staphylococcus aureus*
   - *Streptococcus faecalis*
   - *Mycobacteria phlei*
   - *Sarcina lutea*
   - *Klebsiella pneumoniae*
   - *Proteus vulgaris*

III

CONTENTS

Pure Culture Techniques

In the microbiology laboratory, the testing and identification of microorganisms requires that the microbial specimen being studied be a *pure culture*, a single species of cells free of unwanted or contaminating organisms. Usually many different culture types are associated together in *mixed cultures*. To study a particular microorganism in terms of its special characteristics, the organism must be isolated or removed from the presence of contamination species. In the hospital setting, patient specimens are routinely brought to clinical laboratories for isolation of the infectious agents. Without proper isolation, the identification and antimicrobial susceptibility testing of the infectious agent would not be possible.

Procedures that permit the isolation of a single species from a mixed culture are **pure culture techniques** and involve not only the mechanical separation of microorganisms as in the streak or spread techniques but the use of various media that impose cultural conditions which permit selection and isolation.

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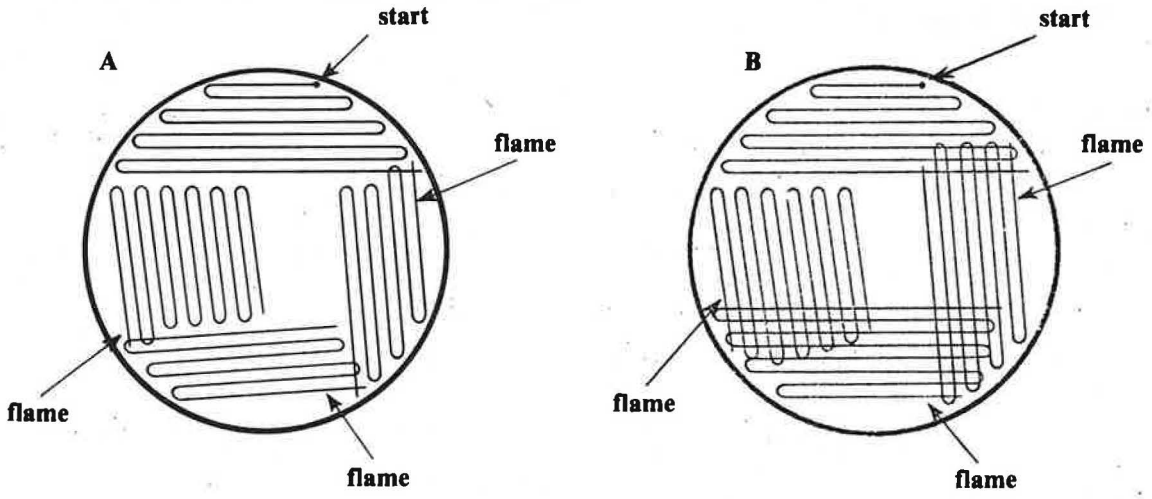
Streak-Plate

The streak-plate technique provides a simple means of separating mixed cultures. Clinically, it or the swab-streak modification is the method of choice. A loop, containing the inoculum, is streaked onto the surface of an agar plate spreading the individual cells apart. The cells multiply during incubation and form isolated colonies of pure cultures. To be absolutely sure of the purity of the isolated colonies, an inoculum should be transferred to about 2 ml of broth or physiological saline, shaken to disperse the cells and streaked on another agar plate. If the colony was pure, all the resulting colonies will appear the same.

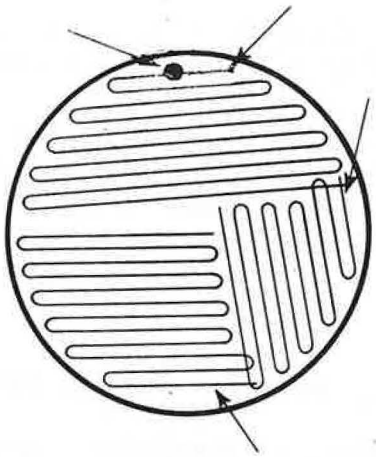


Streak Plate Patterns

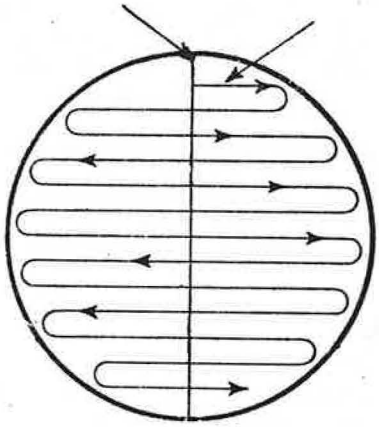
Streaking patterns vary with the concentration of the microorganism, the amount of overlap being the significant difference. Pattern A is used when bacterial concentrations are high and B when they are low.

Swab and Spread Plates

The swab plate and spread plate are alternative methods used to obtain isolated pure cultures. In many instances, specimens to be examined have been taken by swab and contain a high concentration of microorganisms. To directly swab the surface of the plate would result in confluent growth. The method of choice depends on the source of the specimen and usually involves the use of both the swab and the inoculating loop. Two methods, routinely used in clinical laboratories for isolation from throat and urine specimens, demonstrate these considerations.



A. Throat culture method



B. Urine culture method

The spread plate offers a rapid means of spreading a liquid inoculum over the surface of an agar plate. One loopful to 0.1 ml of the mixed culture is placed on the agar plate surface. A glass bent-rod is sterilized by placing the end in alcohol and igniting the tip. When cool, the bent-rod is used to spread the inoculum by rotating it around the surface of the plate.

Materials:

— Broth working cultures of assigned organisms.
— Two (2) Nutrient agar deeps
— Two (2) Petri plates
— One (1) sterile swab
— One (1) bent-rod

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POUR PLATE

The pour plate is an effective means of distributing a bacterial suspension evenly throughout an agar plate. While it may produce isolated colonies, depending on the concentration of bacterial cells, it is not typically used as a pure culture technique. It has many applications in research and industrial laboratories where procedures that determine the number of viable bacterial cells in various products, such as food or cosmetics, are required.

A heavily inoculated pour plate provides an excellent surface on which to test bacterial reactions to antibiotics, disinfectants, chemicals, etc.

Pour plates may be prepared using either of two methods.

1. A measured amount of the bacterial suspension is placed directly in a Petri plate. A melted nutrient medium, cooled to 45°C is poured into the plate, the cover replaced, and the plate gently rotated to achieve uniform distribution of the microorganisms; ***or***
2. The bacterial suspension is placed in a melted nutrient medium that has been cooled to 45° C and the tube is rolled between the palms of the hands for thorough mixing, and then poured into a Petri plate.

Materials:

— Broth working cultures of rhe assigned organism.
— One (1) nutrient agar deep.
— One (1) Petri plate
— One (1) pipet (1 ml).
— Small beaker of 70% alcohol per desk
— Sterile paper disks (1.5 cm diameter)
— Disinfectant (desk wash)

Method:

1. Melt the agar deep and place it in the 45°C water bath.
2. Select one of the methods outlined above.
3. Using 0.5 ml of the bacterial suspension, prepare one pour plate. Allow it to solidify completely.
4. Dip the forceps into the alcohol and ignite to sterilize.
5. Pick up a sterile paper disk with the forceps and touch the edge of the disk to the disinfectant, allowing it to absorb the disinfectant.
6. Lift the cover of the pour plate and place the saturated disk in the center of the agar surface. Close the cover.
7. Incubate, inverted, at 21°C until the next laboratory session.
8. Examine the plate. Note the distribution of the colonies on and in the agar. The colonies within the agar will be lens-shaped. Which of the two methods provides the best distribution of colonies? What effect did the saturated disk have on the development of colonies about it?

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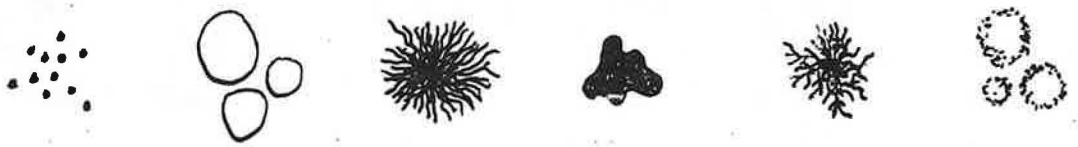
CULTURAL CHARACTERISTICS

When microorganisms are cultured on a variety of media, they exhibit unique and identifiable *cultural characteristics*. In the classical method of identifying microorganisms, it is customary to determine these characteristics by examining the growth of the organism on nutrient agar plates and slants and in nutrient broth and nutrient gelatin. In this experiment, the cultural characteristics of the assigned bacterial species will be examined and described using the following illustrated descriptive terms:

CULTURAL CHARACTERISTICS

Nutrient Agar Plates. Well isolated colonies are evaluated in the following manner:

1. Size: Pinpoint, small, moderate, or large.
2. Pigmentation: Color of colony.
3. Form: The shape of the colony.

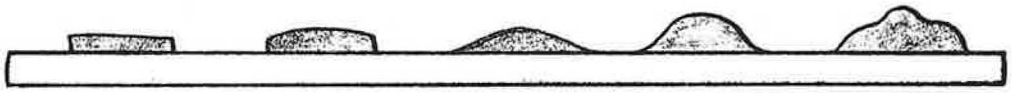


Punctiform **Circular** **Filamentous** **Irregular** **Rhizoid** **Granular**

4. Margin: The appearance of the outer edge of the colony.

Entire *Undulate* *Lobate* *Erose* *Filamentous* *Curled*

5. Elevation: The degree to which colony growth is raised on the agar surface.



Flat **Raised** **Convex** **Pulvinate** **Umbonate**

Materials:

- Broth and slant cultures of the assigned organisms.
- One (1) nutrient broth
- One (1) nutrient gelatin deep
- One (1) nutrient agar deep
- One (1) nutrient agar slant
- One (1) Petri plate

Methods:

A. Transfer of a slant culture to a nutrient gelatin deep

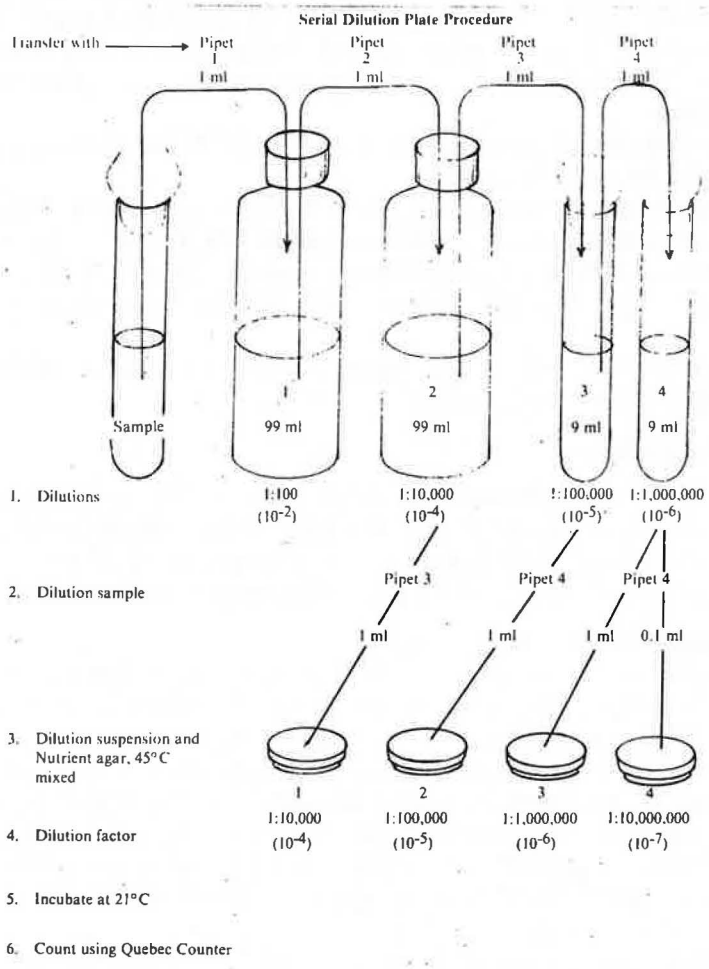
1. Pick up the slant culture and the nutrient gelatin deep and hold in the left hand.
2. Flame the inoculating needle.
3. Remove the cotton plugs from the tubes and hold them between the fingers of the right hand. Sterilize the mouths of the tubes.
4. Hold the tubes almost parallel to the desk top to reduce the possibility of air-borne contaminants.
5. Touch the needle to the medium in the slant culture tube to be sure it is cooled and then touch the culture mass. Remove the needle without touching the sides of the tube.
6. Insert the needle into the nutrient gelatin deep by making a single stab into the center of the media to within 2 cm of the bottom of the tube. Remove the needle along the entry line. Do not stir the medium.
7. Sterilize the mouths of the tubes and replace the cotton plugs. Sterilize the inoculating needle and return it to the rack.
8. Label the nutrient gelatin deep and incubate, inverted, at 21°C until the next laboratory. Do not shake the tube as the pattern of hydrolysis will be disturbed.
9. Log the inoculation.

B. Transfer of a slant culture to a nutrient agar slant

1. Follow instructions 1 through 6 of the previous inoculation procedure.
2. Insert the needle to the base of the slanted portion of the medium and gently draw the needle along the surface making a long, single streak.
3. Sterilize the mouths of the tubes and replace the cotton plugs. Sterilize the inoculating needle and return it to the rack.
4. Label the nutrient agar slant and incubate at 21°C until the next laboratory.
5. Log the inoculation.

SERIAL DILUTION-PLATE FOR VIABLE CELL COUNTS

Many methods have been developed to determine the number of bacterial cells in substances such as food, water, various commercial products, and the environment. These include direct microscopic counts, electronic counters, measurements of bacterial metabolic activity, estimation of dry weight, turbidimetric measurements of increases in cell mass, and the serial dilution plate method. The major disadvantage to most of these is that the total counts include both dead and living cells. Many studies, particularly those involving sanitary and, in some cases medical evaluations, require the determination of the numbers of viable cells. This may be accomplished by using the serial dilution plate method. The procedure involves precise serial dilutions of a bacterial suspension to reduce the number of organisms to a point where they might be plated and effectively counted. Aliquots of the diluted suspensions are plated out on a suitable nutrient medium using the pour plate technique. The viable cells produce colonies on and in the medium and may be counted on the *Quebec colony counter.*



Plates suitable for counting must contain between 30 to 300 colonies to be statistically significant. The total viable cell count of the sample is obtained by multiplying the number of colonies per plate by the dilution factor, which is the reciprocal of the dilution.

Number of cells per ml = number of colonies X dilution factor

Example:

1. **Colonies per plate = 75**
   Dilution factor = 1:1,000,000 ($1:1X10^6$)
   75 X 1,000 = 75,000,000 cells per ml

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The nutrient formulae for the media are based on the organic and inorganic requirements of specific microorganisms or on the basis of the function a specific medium is to serve. In this experiment, two types of media will be prepared: synthetic media designed to test the minimal growth requirements of certain bacteria and a general purpose medium, nutrient agar, formulated to satisfy the requirements of a wide variety of heterotrophic bacteria.

Materials:

— Agar-agar
— Ammonium phosphate
— Glucose
— Magnesium sulfate
— Potassium chloride
— Peptone (or other intermediate product of protein digestion)
— Dehydrated nutirent agar
— Graduated cylinder (1 liter)
— Beaker (1 liter)
— Stirring rod
— Distilled water
— Hot plate
— Wire basket
— Fifteen (15) test tubes and cotton plugs

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Minimal Growth Requirements

Microorganisms have the same basic requirements for the synthesis of complex organic compounds and for the performance of life functions as all living organisms. However, there is great diversity in the compounds they can use. Some use carbohydrate (sugar and starches) as their source while others use ammonium phosphate, potassium nitrate, and other inorganic nitrogen compounds.

To determine the minimal growth requirements for nitrogen, for example, the bacterium is provided with media containing all its nutritional requirements but nitrogen. Various nitrogen compounds, or the like are added signle to determine if growth can occur. The presence of growth indicates the nutritional requirement or enzymatic capabilities of the bacterium.

Materials:

- **Slant cultures of:**
  - *Bacillus subtilis*
  - *Rhodospirillum rubrum*
  - *Pseudomonas fluorescens*
  - *Sacina lutea*
- *Four (4) Petri plates*
- *One (1) deep of each medium prepared during the last laboratory:*
  - *Agar-agar*
  - *Agar-minerals*
  - *Agar-minerals-carbohydrate energy*
  - *Agar-minerals-carbohydrate energy-organic nitrogen*

Methods:

1. Melt and pour one plate of each of the four minimal growth test media. Allow to solidify completely.
2. Divide the bottom of the plate into four sections and label each section with the name of one of the four test organisms.
3. Inoculate the corresponding sections of each plate with the appropriate organism.
4. Incubate at 21°C until next laboratory session.
5. Observe the amount and type of bacterial growth on each plate. Explain the results in relation to bacterial nutrition. Explain the nutritional requirements of an organism that grows poorly on all four media.

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pH Indicators and Controls

One of the most important factors affecting enzymatic action is pH. Enzymes have optimum pH ranges in which biochemical reactions occur. Above or below this optimum pH, reactions are slowed down or stopped completely. Though the acidophiles and alkalophiles tolerate extremes in pH, most bacteria exhibit maximum growth between 6.5 and 7.5. As this narrow pH limit about neutral is beneficial for the growth of most microorganisms, the pH of laboratory medium is frequently adjusted to this range.

As microorganisms grow within a medium, their metabolic wastes often cause pH changes that slow the rate of chemical reactions thereby affecting the growth rate and, ultimately, survival. Because of such changes, it is difficult to keep the pH of culture media constant during growth. **Buffers**, compounds that tend to prevent marked changes in pH, are added to retard these pH shifts.

The following experiment is designed to demonstrate the buffering effect of several solutions and the function of pH **indicators**, dyes which exhibit specific color changes in the presence of acids or bases. Phenol red is frequently used in microbiology laboratories. It is red in solutions with a pH of more than 6.9 and yellow in acidic solutions with a pH of less than 6.8.

Materials:

— 0.1 N HCl and 0.1 N NaOH solutions
— 0.2 M K_2HPO_4 and 0.2 M KH_2PO_4 buffer solutions
— 0.02 percent phenol red indicator solution
— One (1) nutrient broth
— Four (4) test tubes
— Pipets: 10 ml (4); 1 ml (1)
— Distilled water

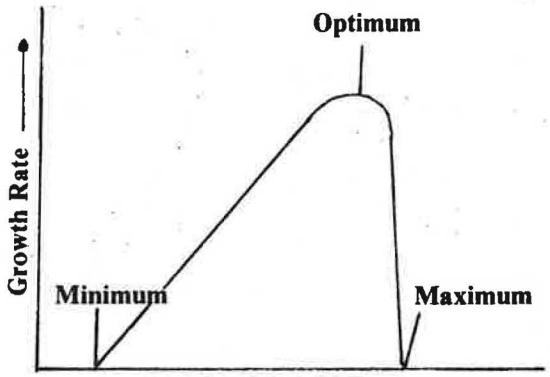
Methods:

1. Work in teams of two (2) members with one set of reagents per desk. Aseptic techniques are not required for this study.
2. Number four (4) test tubes 1 through 4 and set in a rack.
3. Pipet 5 ml distilled water into tube 1. It is the control.
4. Pipet 1 ml of K_2HPO_4 buffer solution into tube 2.
5. Pipet 1 ml of KH_2PO_4 buffer solution into tube 3.
6. Pipet 5 ml of nutrient broth into tube 4.
7. Pipet 4 ml distilled water into tubes 2 and 3.
8. With a 1 ml pipet, add 10 drops of phenol red indicator solution into each of the four tubes and mix well.
9. Record the initial color of each tube.
10. Using a separate pipet for **acid solutions** (HCl) and **basic solutions** (NaOH), pipet 1 ml of HCl into tube 1, mix, and note color change.
11. Add 2 ml of NaOH to tube 1, mix and note the color change.
12. Into tube 2, add HCl one drop at a time with mixing until you get a color change. Record the number of drops required.
13. Into tube 3, add NaOH one drop at a time with mixing until you get a color change. Record the number of drops required. Explain what has occurred in each of the three tubes. Would you add an acid or a base to tube 4, the nutrient borth, to test its buffering power?
14. Into tube 4, add your choice one drop at a time with mixing until you get a color change. How many drops were required to change the color in this solution? Explain. What was the purpose of the indicator?

Temperature Relationships

The growth and survival of microorganisms are greatly influenced by the temperature of the environment. Temperature affects living organisms in either of two ways: (1) with an increase in temperature, chemical and enzymatic reactions occur at an increase rate; synthesis and growth are faster or (2) cellular components, proteins and nucleic acids, are sensitive to high temperature and can become inactive. Therefore, as the temperature increases, metabolism and growth increase up to a lethal point where cell function ceases.

Within the temperature range of the species, there is a minimum temperature below which no growth occurs, an optimum temperature with rapid growth, and a maximum temperature above which life functions cease. These are the **cardinal temperature points** and are characteristic for each organism though they are modified by such environmental factors as pH and nutrition.



Cardinal Temperature Points

Materials:

— Broth working culture of your assigned organism
— Four (4) nutrient agar slants

Methods:

1. **Label each of the slants with the name of your culture and the temperature of incubation (4, 21, 37, 60 degrees C).**
2. **Inoculate the tubes and incubate at the appropriate temperatures until next laboratory.**
3. **Observe the amount of growth and classify your organism as to its range of temperature tolerances. If an organism grew at 20°** C, how would you determine experimentally whether the organism was a psychrophile or a mesophile?

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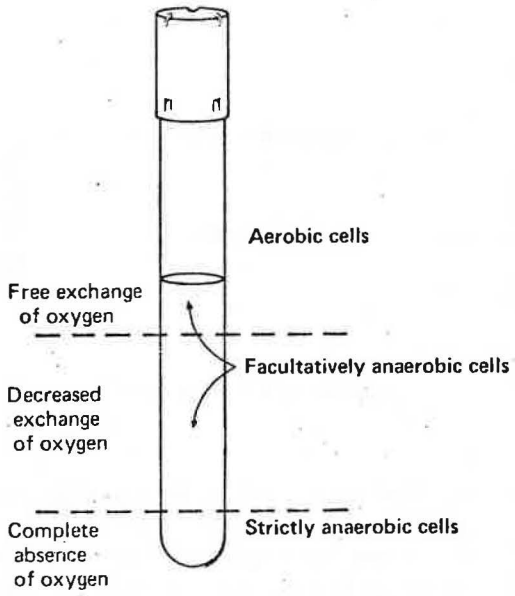
Atmospheric Oxygen Requirements

Bacteria are not strictly aerobic or anaerobic, but demonstrate considerable variation in their responses to gaseous oxygen. They are typically classified into three major groups as illustrated:



Agar Deep Culture

While most aerobes and facultative anaerobes are easily cultivated in the laboratory, obligate anaeorbes require special procedures for recovery, cultivation, and identification. Certain of these procedures have only recently been developed and have greatly increased our knowledge and appreciation of the importance of anaeorbes in infectious diseases. Anaerobes make up more than 50 percent of the total population in many areas of the body and are most likely involved in infections in the blood, abscesses, deep wounds, spinal and synovial fluids, and contaminated food. The few aerobes and facultative anaerobes that are indigenous or parasitic to humans, such as the coliforms *Escherichia* and *Aerobacter*, require high concentrations of carbon dioxide for growth. Such carbon dioxide requiring bacteria are called **capneic**,

The following four (4) methods will be used to evaluate the oxygen and carbon dioxide requirements of selected bacteria:

GasPax Method consists of a jar with alid that is clamped down, a catalyst located in the lid, and a replaceable envelope congaining hydrogen-generating chemicals that are activated when water is added to the envelope. When the water is added to the envelope, any oxygen in the jar reacts with the hydrogen gas and in the presence of the catalyst produces water. The system also contains a methylene blue indicator strip which is colorless under anaerobic conditions.

Methods:

1. **Each team of our will be assigned four bacteia. Each member will culture only one of the bacteria. Have a Working Culture of your assigned Stock Culture ready for inoculations.**
2. **Melt ten (1) nutrient agar deeps and cool to 45°C** in the water bath.
3. Label all plates and tubes.

A. **GasPax and Candle Jar Methods**

1. Pour and cool two (2) Petri plates per team.
2. Using a wax pencil, mark the bottom of the two plates into four (4) sections and label each section with the name of one of the four bacterial cultures.
3. Inoculate your culture into the appropriate section on each plate and have your team members do the same until all sections are inoculated.
4. Place the inoculated plates in the GasPax and Candle jars. These will be incubated at 21°C and returned to you during the next laboratory.

B. **Thioglycollate Broth**

1. Inoculate the thioglycollate broth with one loopful of your culture.
2. Incubate at 21°C until the next laboratory.

C. **Shake-Culture Method**

1. Inoculate the melted and cooled agar deep with one loopful of your culture.
2. Shake thoroughly. Do not permit the cotton plug to become moist.
3. Using a **working culture** of your Stock Culture, inoculate one loopful into a melted and cooled agar deep and shake thoroughly.
4. Incubate both tubes at 21°C until next laboratory session.

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Selective and Differential Media

The isolation of a particular organism may involve the use of several media until a pure culture is finally obtained. For example, to isolate a Gram-negative bacillus, a medium that inhibits Gram-positive but not Gram-negative organisms might be used first. Once the Gram-negative colonies are obtained they might be transferred to another selective media that inhibits certain of the Gram-negative forms or a differential medium that permits distinguishing organisms on the basis of macroscopic colony characteristics. When differentiating bacteria as to their species classification, biochemical reactions become important. Differential media distinguish among bacteria on the basis of chemical reactions as well. Media containing a pH indicator will change color when acid or alkaline metabolic wastes are produced due to the enzymatic activity of certain bacterial species.

Materials:

- **Broth cultures of:**
  - *Alkaligenes viscolactis*
  - *Sarcina lutea*
  - *Escherichia coli*
  - *Bacilus subtilis*
- One (1) nutrient agar deep
- One (1) sodium chloride agar deep
- One (1) phenol red dextrose agar deep
- One (1) Crystal violet agar deep (1:100,000 dilution)
- Four (4) Petri plates

Methods:

1. Work in teams of four (4) members.
2. Melt and pour one plate each of the four types of media.
3. Divide the bottom into sections and label each section.
4. Inoculate the corresponding section of each plate with the appropriate organism.
5. Incubate at 21°C until next laboratory.
6. Observe the plates and explain the reactions. Which of the four media was selective? Differential? How would you further isolate these organisms if these media did not produce a pure culture of the desired organism?

27

Primary Isolation Media

Most clinical specimens contain a mixed flora of microorganisms. Depending on the sourcee and the expected infectious agents, numerous media, called **primary isolation media**, are inoculated. These serve several purposes: (1) to determine the predominating species; (2) to differentiate species, and (3) to selectively encourage the growth of the suspected pathogens.

The physical requirements of organisms (temperature, pH, and oxygen) function as selective agents as well and are consistently used to isolate specific microorganisms.

Materials:

- **Simulated fecal suspension (SFS):**
  Escherichia coli
  Pseudomonas aeruginosa
  Staphylococcus epidermidis
- One (1) prepared blood agar plate
- One (1) Eosin methylene blue agar deep (E.M.B.)
- One (1) Mannitol salt agar deep (M.S.A.)
- One (1) MacConkey agar deep
- Two (2) Trypticase soy agar deeps
- Five (5) Petri plates
- Three (3) sterile water blanks
- Three (3) sterile swabs
- One (1) urine specimen cup
- One (1) sterile towelette
- *Difco Manual* or *BBL Manual of Products and Laboratory Procedures*
- *Demonstration streak plates of:*
  Escherichia coli
  Staphylococcus aureus

Methods:

A. Primary Isolation of Microorganisms

1. **Divide four (4) Petri plates and the blood agar plates into four (4) sections and label them: nose, throat, urine, and S.F.S.**
2. **Melt the agar deeps and pour the E.M.B., M.S.A., MacConkey, and one of the trypticase-soy agar deeps into the previously section Petri plates. The remaining melted trypticase-soy deeps should be kept in the 45° C water bath** ready for preparation of a urine specimen pour plate.
3. Swab your throat and inoculate each of the five sectioned plates in the proper area.
4. Repeat using a nose swab and the S.F.S.
5. Collect a mid-stream urine specimen. A sterile towelette is provided.
6. Prepare a serial dilution plate of approximately 1:1000. The U.S. Public Health Service uses a bacterial count of 10_5 per ml as an index of significant bacteriuria.
7. Centrifuge a urine sample for three minutes and decant the supernatant in the container provided. Do keep it covered.
8. Pick up a sample of the sediment in the tube, and inoculate the plates. Do not swab the pour plate.
9. Prepare a Gram's stain and determine the cell shape and arrangement of any bacteria present. The larger cells with nuclei are epithelial cells.
10. Examine the demonstration plates of M.S.A. and E.M.B.
11. Incubate the plates at 37° C for 24—48 hours. Describe the amount of growth and the colonies on each of the primary isolation media, note any changes in the media and indicate the selective or differential qualities of the media by comparing them with the growth on the trypticase-soy plate. Further studies may be done using the specimen cultures. Discuss these with your instructor.

UNIT IV
BIOCHEMICAL CHARACTERISTICS OF MICROORGANISMS

CONTENTS

Biochemical Characteristics of Microorganisms

All of the activities of the microbial cell are mediated by species specific enzymes. In many cases, **exoenzymes** are secreted into the environment where they modify macromolecules releasing smaller molecules that may enter the cell. There, **endoenzymes** utilize them as energy sources or building blocks for synthesis of cell constituents and in the process, metabolic wastes are produced and released into the environment. Most tests done in microbiology laboratories measure these activities, determining the cell's ability to produce specific enzymes, metabolites, and end products. The identification and classification of bacteria is to a considerable extent based on such changes produced by pure cultures growing in or on various differential media.

The following experiments will use routine methods to determine the biochemical characteristics of the assigned bacterial cultures. These bacteria are representative types and will serve as the basis for the identification of unknown organisms.

The biochemical characteristics of microorganisms and the media used are outlined below. Review the purpose of these media in either *Difco Manual* or *BBL Manual of Products and Laboratory Procedures* and the characteristic reactions of your bacterium in *Bergey's Manual of Determinative Bacteriology.*

BIOCHEMICAL ACTIVITY	MEDIA	REAGENTS/INDICATORS
I Exoenzymatic Activities		
Starch hydrolysis	Starch agar plate	Iodine
Lipid hydrolysis	Tributyrin agar plate	Neutral red
Casein hydrolysis	Casein agar plate	None
Gelatin hydrolysis	Nutrient gelatin deep	None
	Gelatin agar plate	Picric acid
II Endoenzymatic Activities		
Carbohydrate fermentations	Fermentation tubes	Phenol red
	Fermentation disk plate	Phenol red
Litmus milk reactions	Litmus milk tubes	Litmus
Nitrate reduction	Tripticase-nitrate broth	Sulfanilic acid, alpha-naphthylamine, and zinc
Urease production	Urea broth	Phenol red
	Nutrient broth	Urease test tablets
Oxidase production	Trypticase-soy plate	Oxidase test paper
Catalase production	Trypticase-soy plate	Hydrogen peroxide
Battery Tests		
Hydrogen sulfide production	S.I.M. deep	Ferrous sulfate
	T.S.I. deep slants	Ferrous sulfate
Indole production	S.I.M. deep	Kovac's reagent
Motility	S.I.M. deep	None
Glucose fermentation	MR-VP	Methyl red
Acetylmethylcarbinol production	MR-VP	Barritt's reagent
Citrate utilization	Simmon's citrate slant	Brom thymol blue
Carbohydrate fermentations and H_2S production	T.S.I. deep slants	None

NOTE:

You will be using a variety of media, some of which look very similar. Be sure to label the tubes as you pick them up at the media cart.

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Hydrolysis of Polysaccharides, Proteins, and Lipids

The macromolecules of starch, lipids, and proteins are insoluble and cannot be taken into the cell directly. Bacterial exoenzymes must be secreted to hydrolyze these molecules into smaller units: starch is broken down to monosaccharides and disaccharides; lipids to fatty acids and glycerol, and proteins to amino acids. These molecules can then pass through the cell membrane and be utilized. The hydrolysis of these substrates may be demonstrated by changes in the media.

Materials:

- **Slant cultures of:**
  Bacillus subtilis
  Escherichia coli
  Proteus vulgaris
  Pseudomonas fluorescens
  Working cultures of assigned organisms
- Four (4) starch agar deeps
- Four (4) Tributyrin nutrient agar deeps with neutral red indicator
- Four (4) Frazier's gelatin agar deeps
- Twelve (12) Petri plates
- Dilute iodine solution
- Picric acid

Methods:

1. **Work in teams of four (4) members. Each member will use one of the prepared cultures and his/her working culture.**
2. **Divide the plates into two (2) sections and label fully including the name of the medium.**
3. **Melt and pour the media into the proper plate, allowing them to solidify completely.**
4. **Inoculate each section of each of the three plates with the appropriate organism, making one heavy streak.**
5. **Incubate at 21°C** until next laboratory.
6. Flood the gelatin agar plate with picric acid solution and wait about 15 to 30 minutes. Clear zones around the colonies indicate a positive test for proteolytic enzymes which hydrolyze gelatin.
7. Flood the starch agar plate with dilute iodine. Iodine turns starch blue-black. Starch hydrolysis is indicated by the absence of the color reaction. A clear area around a colony is a positive reaction for starch hydrolysis.
8. Examine the trybutyrin agar plate. The neutral red indicator turns deep red in the presence of acid. This color change indicates those colonies that have hydrolyzed the lipid to glycerol and fatty acids.

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Carbohydrate Fermentations

Most aerobic and anaerobic microorganisms use carbohydrates as their primary source of energy. Monosaccharides and disaccharides are oxidized by ordered sequences of enzymatic reactions or biooxidative pathways. By means of **cellular respiration**, with or without oxygen, or **fermentation**, the energy within the chemical bonds of the carbohydrate is released (for use by the cell) and various end-products are the result. Bacteria differ greatly in the end-products they produce from a given sugar. Some produce both acid and gas, while others produce only acid. Others produce neither.

Two methods will be used to determine the fermentative activity of bacteria: fermentation tubes and fermentation disks. Fermentations usually occur within 24 to 48 hours after inoculation. Extended incubation permits other enzymatic reactions to mask those of fermentation producing false negatives or positives. The production of acid will cause the indicator, phenol red, to turn yellow. The gases that may be produced within the fermentation tubes will be trapped within the inverted Durham tube.

Materials:

— Slant cultures of:
  Bacillus subtilis
  Escherchia coli
  Staphylococcus aureus
  Alcaligenes viscolactis
  Working cultures of assigned organisms
— Eight (8) fermentation tubes of:
  lactose
  glucose (dextrose)
  sucrose
— Four (4) nutrient agar deeps containing 0.025 g phenol red per liter
— 0.05 percent aqueous concentrations of selected sugars
— Four (4) Petri plates
— Forceps and alcohol

Methods:

A. Fermentation Sugar Disc Method

1. Work in teams of four (4) members. Each member will use one of the prepared cultures and his/her working culture.
2. Label the fermentation tubes as you pick them up from the media cart.
3. Using 1 ml of your working culture, prepare a phenol red nutrient agar pour plate. Allow it to solidify completely.
4. Divide the plates into six (6) sections and label each section with a code number for each sugar to be tested. Record your code.
5. Using sterile forceps (dipped in alcohol and ignited), pick up and moisten a sterile disc in one of the sterile sugars. Place it in the proper section of the plate.
6. Repeat procedure 5. for each of the sugars.
7. Incubate the plates for 48 hours at 21° C. Observe and record the reactions at 24 hours and again at 48 hours.

B. Fermentation Tube Method

1. Inoculate a lactose, glucose, and sucrose fermentation tube using one of the cultures prepared for your team.
2. Incubate for 48 hours at 37° C. Observe, record the reactions at 24 hours and again at 48 hours. Do not discard the tubes at this time.

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Litmus Milk Reactions

Litmus milk is an excellent media for growth and differentiation as it will support the growth of a variety of microorganisms. It contains the milk sugar, lactose; the milk proteins, casein, lactoalbumin, and lactoglobulin; vitamins, and minerals. Litmus is added as an indicator as well as a reducible dye. As an indicator, it turns pink in the acid condition and more deeply purple in the alkaline condition. As a reducible dye, it loses its color and becomes creamy white.

Because of the nature of the medium, a variety of reactions may occur:

1. **Lactose fermentation** occurs when an organism uses lactose as a carbon source of energy. The metabolic pathway is through glycolysis to lactic acid. Lactic acid production is detected when litmus turns pink (at approximately pH 4) and an acid curd forms in the milk. Acid curds are firm and remain in place when the tube is tilted.
2. **Reduction of litmus** is due to the oxidative activities of the bacterium and is indicated by the loss of litmus color. This is most apparent when a pink acid curd begins to turn white.
3. **Gas formation,** as an end-product of lactose fermentation, may include carbon dioxide and hydrogen. Evidence of gas may be seen as breaks or fissures within the acid curd.
4. **Rennin curd production** will occur when certain bacteria produce enzymes that form an insoluble calcium-casein complex. Unlike the acid curd, rennin forms a soft, semisolid curd that flows slowly when the tube is tilted.
5. **Proteolysis** occurs when some microorganisms, unable to obtain energy by way of lactose fermentation, hydrolyze the milk proteins to their basic units, aminio acids. As the proteins are digested, the medium begins to clear starting at the top of the tube and proceeding downward. In addition, the medium becomes brownish and translucent.
6. **Alkaline Reaction** is also due to the utilization of casein. Ammonia, which is highly soluble in water, and other basic substances accumulate causing the litmus to change from light purple to deep purple.
7. **Ropiness** is the result of the growth of such organisms as *Alkaligenes viscolastis.* The cells become encapsulated due to the highly nutritive environment and form a slimy growth in the medium. This "ropiness" can be detected by testing the medium with an inoculating loop and observing long "ropes," or masses of cells.

Materials:

— **Broth cultures of:**
  Escherichia coli
  Alcaligenes viscolactis
  Streptococcus lactis
  Pseudomonas aeruginosa
  Clostridium sporogenes
  Working culture of the assigned organisms
— Nine (9) tubes of litmus milk

Methods:

1. Work in teams of four (4) members.
2. Label each of the tubes with the name of the organism, your initials and the date.
3. Inoculate the tubes with the appropriate organism.
4. Incubate at 37°C. because these reactions become apparent at different time intervals, observe the tubes at 48 hours and again after five (5) days.

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Nitrate Reduction

Microorganisms are versatile in their ability to utilize different sources of nitrogen. Some are able to convert atmospheric nitrogen into forms that are precursors to organic molecules, while others degrade organic wastes releasing essential materials into the environment. Intermediate to these is a wide range of microbial activities that not only make nitrogen available to microorganisms but evolve forms of nitrogen useable by other organisms. Because of their diverse enzymatic capabilities, microorganisms play an important role in the nitrogen cycle by maintaining a flow of nitrogen throughout the biosphere and providing all forms of heterotrophic life, ourselves included, with essential sources of nitrogen.

Bacteria differ in their ability to use a specific form of nitrogen, for example nitrates. Some can reduce nitrates to nitrities. Others can reduce nitrates to the ammonia and gaseous nitrogen. Still others do not reduce nitrates at all. These various reactions provide another means of differentiation and identification of bacterial types.

The reduction of nitrate and the subsequent reduction of nitrite can be detected by growing a bacterium in nutrient broth to which potassium nitrate has been added. One of the following results may occur depending on the enzymatic capabilities of the bacterium:

1. The nitrate may remain unaltered.
2. Nitrate may be reduced to nitrite.
3. Nitrate may be reduced to nitrite and then further reduced to ammonia or free nitrogen.

Materials:

- **Broth cultures of:**
  - *Excherichia coli*
  - *Pseudomonas aeruginosa*
  - *Proteus vulgaris*
  - *Bacillus subtilis*
  - Working cultures of assigned organism
- Nine (9) Trypticase-nitrate broths
- Nitrite reagents:
  - Sulfanilic acid solution
  - Dimethyl-alpha-naphthylamine solution
- Zinc powder

Methods:

1. Work in teams of four (4) members.
2. Label and inoculate tubes of trypticase nitrate broth with the appropriate bacterium.
3. Incubate at 37°C until the next laboratory session.
4. Layer 1 ml of each of the two nitrite reagents onto the surface of the medium in each tube. These reagents test for the presence of nitrite. If the test is positive for nitrite, a distinct red color will appear, indicating nitrate has been reduced to nitrite. If the test is negative for nitrite, it must be considered a **presumptive negative** for the following reasons:
   a. Nitrate may not have been reduced by the bacterium.
   b. Nitrites were produced but the bacterium was also able to reduce the nitrites to ammonia and nitrogen.

   In either case, no nitrites would be present in the medium. To the presumptive negative tubes, add a trace of zinc powder. Zinc reduces nitrates to nitrites. If a red color appears now, it means the nitrate remained unaltered by the bacterium and available for reduction by the zinc. The test is a **confirmed negative**. If no color change occurs, it means that the nitrate has been reduced by the bacterium to ammonia and/or nitrogen.

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Urease Production

The interdependence of organisms becomes most evident when examining the physiology of microbes. Many organisms are able to synthesize amino acids from the by-products of carbohydrate and lipid metabolism if they are provided with ammonia as a source of nitrogen. Many other organisms are able to convert various forms of inorganic oxidized nitrogen to ammonia. Some bacteria are able to convert urea, an organic waste product of animal metabolism, into ammonia and carbon dioxide.

$$O=C(NH_2)(NH_2) \xrightarrow[H_2O]{\text{urease}} 2\,NH_3 + CO_2$$

The ability of microorganisms to produce urease and effect urea hydrolysis is used to differentiate the genus *Proteus* from other inteic Gram-negative organisms, especially enteric pathogens such as *Salmonella* and *Shigella* which it closely mimics.

The presence of urease production may be detected using two methods: **urea broth**, containing nutrients, urea, and phenol red and **Urease Test Tablets** which are added to nutrient broth cultures to provide urea and phenol red for a rapid test of urea hydrolysis. As urease positive organisms hydrolyze urea releasing ammonia, the pH of the medium becomes alkaline and the indicator turns deep red.

Materials:

— Broth cultures of:
  Escherichia coli
  Proteus vulgaris
  Working cultures of the assigned organisms
— Six (6) urea broth tubes
— Seven (7) urease est tablets
— One (1) nutrient borth

Methods:

A. Urea Broth Test

1. **Work in teams of four (4) members in both studies.**
2. **Label and inoculate the urea broth tubes.**
3. **Incubate 24 to 48 hours at 37°C.**
4. Examine the urea broth culture tubes and note any changes in the indicator color. An uninoculated urea broth may be used for comparison. Urease production is indicated by a change in color to deep red. No change in color occurs in tubes inoculated with urease-negative organisms.

B. Urea Test Tablet

1. **Aseptically place one urease test tablet in each of the broth culture tubes previously used for inoculations. Do not use the urea broth tubes for this study.**
2. **Place another tablet in a sterile nutrient broth as a control.**
3. **Examine the tubes as in Method A.**

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Oxidase and Catalase Production

Oxidase and catalase are important in the differentiation and identification of certain microorganisms. Both are associated with the electron transport system in aerobic organisms. *Cytochrome oxidase functions in the transport of the hydrogen atom to oxygen resulting in the formation of water by some organisms and hydrogen peroxide by others. All aerobic, as well as some facultative anaerobes and micro-aerophiles, produce cytochrome oxidase. Those organisms tht produce hydrogen peroxide, a substance toxic to cells, also produce catalase,* an enzyme that breaks down hydrogen peroxide to oxygen and water.

$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2\ H_2O + O_2$$

The oxidase test is useful in the differentiation of members of the genera *Neisseria* and *Pseudomonas* which are oxidase-positive and the family Enterobacteriaceae which are oxidase-negative. The catalase test identifies the catalase-negative genera which includes *Streptococcus* and *Clostridium.*

Materials:

— Broth cultures of:
  Escherichia coli **Group 1**
  Pseudomonas fluorescens
  Staphylococcus aureus **Group 2**
  Streptococcus faecalis
  Working cultures of he assigned organisms
— One (1) trypticase-soy agar deep
— One (1) Petri plate
— Three (3) oxidase test disks
— Three (3) capillary tubes
— Small beaker of hydrogen peroxide

Methods:

1. **Divide the Petri plate into three (3) sections and label with the names of Group 1 organisms and your assigned organism.**
2. **Melt and pour a trypticase-soy agar plate.**
3. **Streak each section with the appropriate organism and incubate at 21°C** until next laboratory session.
4. For the oxidase test, place an oxidase test paper on one end of a clean slide and moisten with a drop of distilled water.
5. Transfer a loopful of one culture to the test paper and rub it in thoroughly.
6. After approximately 30 seconds, examine the treated area for color change. A blue color indicates a positive oxidase test.
7. Repeat using each of the other cultures.
8. For the catalase test, place the capillary tube into the beaker of hydrogen peroxide and allow capillary action to fill the tube to about 2 cm.
9. Touch the tip of the capillary tube to one of the cultures on the plate. If the organism is catalase-positive, bubbles will develop within the capillary tube.
10. Repeat the procedure on each of the cultures in the plate. This technique is useful when the culture medium is blood agar. Blood also gives a catalase-positive reaction and therefore could confuse the test reaction.
11. Now, place one drop of hydrogen peroxide on the colonies in each section. The choice of technique is dependent on the medium used, Incidently, hydrogen peroxide is generally a poor antiseptic as it is rapidly broken down by bacterial catalase.

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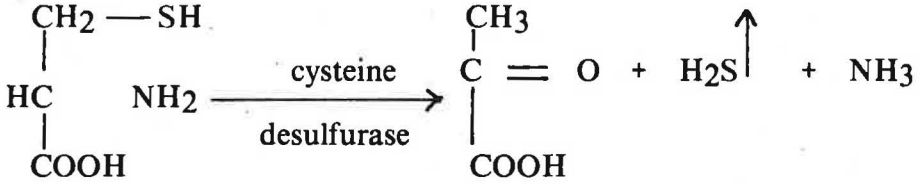
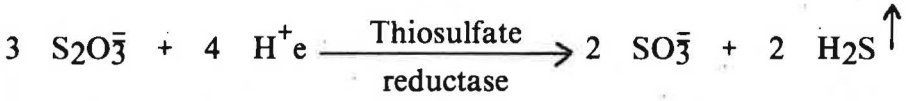
SIM tests

Bacteria are able to use sulfur-containing amino acids and inorganic compounds in the fermentative production of **hydrogen sulfide**. Some bacteria produce the enzyme *cysteine disulfurase*, which removes sulfur from the amino acid, cysteine, during the deamination of cyteine to pyruvic acid, and releases hydrogen sulfide.



Cytseine — Pyruvic acid — Hydrogen sulfide — Ammonia

Hydrogen sulfide is also produced by bacterial action in the reduction of inorganic sulfur compounds such as *thiosulfate.*



Thiosulfate — Sulfite — Hydrogen sulfide

If metallic ions, such as iron, lead, or bismuth, are available, the hydrogen sulfide formed during growth combines with the metallic ions to form a metal sulfide that blackens the medium. Examples of this reaction are seen in cans with spoiled food and in sediments in some ponds. The characteristic odor is that of rotting eggs.

The bacterial production of the enzyme *tryptophanase* converts the amino acid tryptophan into *indole*, pyruvic acid, and ammonia. While indole is a putrefactive waste product, pyruvic acid and ammonia may be used within the cell for energy and protein synthesis. SIM medium is a convenient medium for testing for hydrogen sulfide and indole production as well as motility determination. It contains peptones and thiosulphates as sources of the enzyme substrates, ferrous ammonium sulfate as a source of iron ions which function as a hydrogen sulfide indicator, and sufficient agar to promote anaerobic respiration and determine bacterial motility.

Materials:

— Slant cultures of:
  Aerobacter aerogenes
  Escherichia coli
  Proteus vulgaris
  Bacillus subtilis
  Working culture of the assigned organism
— Eight SIM deeps
— Kovack's reagent

Methods:

1. Work in teams of four (4) members. Each member should use one prepared culture and his/her assigned organism.
2. Label each of the SIM deeps with the name of the organism and your initials.
3. Inoculate each of the organisms into the appropriately labeled tubes using the stab method.
4. Incubate at 21°C until next laboratory session.
5. Observe for hydrogen sulfide production and motility. A black precipitate of ferric sulfide indicates hydrogen sulfide production. The diffusion of growth away from the stab line in this semisolid medium indicates the presence of motile cells.
6. Add about 1 ml of Kovac's reagent. Mix by rolling the tube between the hands and let stand for a few minutes. The presence of indole is indicated by the development of a deep red color within the reagent layer.

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MR-VP TESTS

Glucose fermentation yields three types of end-products: gases, acids, and neutral substances. Though all enteric bacteria ferment glucose as their primary source of energy, the kind and amount of end-products vary according to the enzymatic pathways present in the bacteria. The MR-VP tests are designed to differentiate bacteria on the basis of these fermentative end-products. Bacteria that produce large amounts of **acids** from glucose lower the pH of the medium to below pH 4.5 where, because of the high acid concentration, further growth is limited. Other bacteria initially produce acids but the lowered pH triggers the induction of enzymes that convert the acids into neutral end-products such as *2,3 butanediol* and *acetylmethylcarbinol* causing the pH of the medium to increase to about 6. These differences in enzymatic reactions are the basis for the methyl red (MR) portion of the test.

Methyl red is an indicator with a pH range of 4 to 6 and is red at pH 4 or below and yellow at pH 6 or above. Bacteria that produce acids decreasing the pH to 4 and below turn the media red and are *methyl red-positive*, while those that have a final pH of 6 and above due to the subsequent conversion of the acids to neutral end-products turn the medium yellow and are *methyl red-negative*.

The Voges-Proskauer (VP) test works in conjunction with the methyl red test in that it measures the presence of the neutral end-products *2,3 butanediol* and *acetylmethylcarbinol* (acetoin) from glucose fermentation. Two reagents are used in the VP test to produce a color change: Barritt's reagent (alpha-naphthol) and 40% KOH. Alpha-naphthol reacts with acetoin or 2,3 butanediol in the presence of air and KOH to produce diacetyl. The diacetyl in turn reacts with a guanidine residue of arginine in the broth to produce a rose color.

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Triple Sugar Iron Agar Tests

Triple sugar iron agar is a multipurpose medium which combines tests for the fermentations of glucose, lactose, sucrose, and the production of hydrogen sulfide. It is designed to efficiently differentiate the groups and genera of the Enterobacteriacea, which are are all Gram-negative bacilli that ferment glucose to acid, and to distinguish the Enterobacteriacea from other Gram-negative bacilli.

Triple iron sugar agar is a slant medium prepared with a deep butt. Microorganisms are inoculated into and on the medium by the stab-streak method. There they may metabolize the sugars to acids by the glycolytic process. Phenol red is used as the indicator and turns yellow with acid production. The microorganisms may also metabolize sulfur-containing amino acids or thiosulfate in the medium producing hydrogen sulfide. Hydrogen sulfide combines with the iron ions in the medium to produce visible ferrous sulfide. Most microorganisms can grow on this medium but differ in their enzymatic capabilities to metabolize the sugars and amino acids. These differences result in characteristic changes in the medium:

1. **Alkaline slant and acid butt** indicate that glucose is the only one of the three sugars metabolized. The reasoning for this is:
   - The bacteria preferentially ferment glucose first.
   - The concentration of glucose in the medium is minimal, one-tenth the concentration of the other two sugars.
   - Air is available in the slant and permits the oxidation of the acids to neutral products.
   - The anaerobic conditions within the butt of the tube prevent the oxidation of the acids.
2. **Acid slant and acid butt** indicate that lactose and/or sucrose have been metabolized because:
   - Organisms that metabolize a sugar other than glucose will produce considerably more acid due to the high concentration of these sugars.
   - Oxidation in the slant area does not occur rapidly enough to produce enough neutral end-products to change the pH.
3. **Alkaline slant and alkaline butt** indicate that no carbohydrate fermentation has occurred because:
   - Some microorganisms cannot metabolize any of the sugars in the medium. Their carbon and energy sources are the peptones provided in the medium.
   - Peptones metabolized under aerobic and/or anaerobic conditions produce ammonia and result in an alkaline pH.

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Isolation and Identification of Bacteria in Mixed Cultures

One of the major responsibilites of the microbiologist is the isolation and identification of unknown bacteria. Clinical specimens and samples of food, water, and various products are diligently examined to determine the causative agents of disease and the presence of contaminants. Countless materials are examined in the search for new microbial sources of useful products such as antibiotics, enzymes, vitamins, and solvents. You have now developed sufficient knowledge of isolation and staining techniques and the biochemical activities and characteristics of bacteria to be able to work independently in an attempt to isolate and identify unknown species in mixed cultures.

You will be given a mixed culture containing several bacteria selected from those previously studied as well as a few unfamiliar species. You will be required to isolate and identify each of the bacteria using only that media necessary for identification. In order to help you organize your laboratory tests a **flow chart** will be prepared using the data you have collected from previous tests of your assigned bacterium. The chart will be of assistance in planning the media you will use for the rapid and efficient identification of your unknowns. Keep neat, up-to-date, and accurate records in your log book. Leave a series of pages for each unknown. Do not spread the data on a particular bacterium throughout various pages of the log book.

Materials:

— Mixed culture
— Two (2) nutrient agar deeps
— Two (2) Petri plates
— Media **as requested**

Methods:

1. Prepare two (2) nutrient agar plates. Dry the plates in the incubator to remove excess moisture.
2. Immediately upon receipt of the suspension of your unknown mixture, streak each plate to obtain well-isolated colonies. Incubate one plate at room temperature; the other label with your name and unknown mixture number and place in in the **inverted** position in the tray as provided. This plate will be incubated at 37°C for 24 hours and graded for your streaking technique, then returned to you.
3. After you have streaked your plates, prepare a Gram stain of the unknown mixture. Observe and record the Gram reactions, the cellular forms, and the cell arrangement of the different types of bacteria present.
4. Examine your plates daily for 1 to 3 days after incubation to detect and distinguish each different type of colony present. Select, number, and transfer a bit from representative, well-isolated colonies of each different colony type to a small drop of water on a slide; dry, fix, and Gram stain each. Examine each for Gram reaction, morphology, cell group, and **purity**.
5. When you are satisfied that you have a pure culture of each of the types present, transfer a small amount of each to a nutrient agar slant. Incubate at 37°C for 24—48 hours. Do another Gram's stain to verify the purity of each culture.

UNIT V

CONTENTS

UNIT VI

UNIT VII

V

V CONTROL OF MICROORGANISMS

There are few places where microorganisms cannot be found and many places where it is essential that they not be found. While many bacteria are beneficial and, in fact necessary, others are pervasive, and can be destructive and lethal. The effective control of such bacteria is necessary to prevent or treat diseases, inhibit the spoilage of food, industrial products, and exclude their contaminating effects in precise research and development, etc. Most methods of control involve physical and chemical agents that adversely affect bacterial structures and functions, thereby inhibiting their growth or killing them. These measures include the use of heat, radiation, antiseptics, disinfectants, antibiotics, and many other agents.

Though the mode of action of the different physical and chemical agents may vary, they all cause damaging effects to the: (1) **cell wall** by lysis or inhibition of cell wall synthesis, (2) **cell membrane** by lysis or impairment of the mechanisms for taking up solutes, (3) **cytoplasm** by denaturing cellular proteins, (4) **enzymes** by inactivation or coagulation, and/or (5) **nucleic acids** by affecting their structure and function.

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Determination of Thermal Death Point and Thermal Death Time

in spite of the diversity among microorganisms as to their range of temperature tolerances, extremes in temperature can be used to control bacterial growth. Low temperatures, even those of lyophilization, will inactivate enzymes, producing a bacteriostatic effect, while high temperatures irreversibly denature cellular and enzymatic proteins and are, therefore, bactericidal.

The rate at which a bacterial population is killed by heat is determined by a number of factors: (1) the particular species; (2) the growth phase of the population; (3) the concentration of the population; (4) the presence of spores; (5) the time of exposure; (6) the intensity of the heat; (7) the nature of the substrate; and (8) the presence of other control agents; *i.e.,* hydrogen ion concentration. These factors are also applicable to the control of bacteria by other physical and chemical agents as well.

In order to compare the relative susceptibility of different organisms to elevated temperatures it is necessary to determine their response under various conditions using precise scientific procedures. Two methods are routinely used: the **Thermal Death Point (TDP),** the lowest temperature at which a standard suspension of a given bacterial species is sterilized in ten minutes, and the **Thermal Death Time (TDT),** the shortest time required to sterilize the suspension of a certain species at a given temperature. Because of the number of other factors involved, the numerical values of TDP and TDT show some variability and are useful only as guidelines. These principles are the basis of microbial control in the preservation of food, pasteurization of milk, preparation of sterile media and hospital supplies, and decontamination of materials.

This experiment will determine the TDT of five (5) bacterial cultures: a Gram-positive cocci at high and low concentrations, a bacillus in the vegetative and spore-forming stages of growth, and a Gram-negative bacillus. By exposing each culture to heat of a specific intensity and taking samples at specified intervals, it is possible to determine the TDT of each culture. The determination of species differences may be made by comparing the TDT of the three different bacteria. The TDP will be determined by exposing each bacterium to a range of heat intensities for ten (10) minutes.

Determination of Thermal Death Point

1. **Label four (4) nutrient broth tubes with the name of the organism, and the growth stage or concentration if applicable. Label the exposure temperatures: 100°C; 90°C; 80°C; 70°C, and 60°C.**
2. Place five (5) nutrient broth tubes in the water bath to be heating.
3. Transfer 1 ml of the test organism to one of the heated tubes in the water bath. Record the time and discard the pipet.
4. In exactly ten (10) minutes, transfer one loopful of the heated culture to the tube labeled 100°C.
5. Turn off the heat and cool the water to 90°C by slowly adding water and mixing after each addition. If you cool it too far, turn the heat on once more. Remove some of the water if necessray.
6. Repat procedures 3 and 4, placing the inoculum in the 90°C tube.
7. Cool the water bath to 90°C and repeat the process. Continue, repeating the process at 70°C and 60°C, exposing the culture for only ten (10) minutes each time.
8. Incubate the inoculated tubes at 37°C until next laboratory session.

Use the following formats to record your results. Record the growth as positive (+) or negative (—).

TDT

Time began_____ Time began_____

Bacterial Species	Control	63°C					100°C			
		1 min	5 min	10 min	15 min	30 min	1 min	2 min	3 min	5 min

NOTE:

The approximate TDT is the first time showing negative growth in the table. An accurate TDT would have to be done with much shorter time differences.

TDP

Bacterial Species	Control	10 minutes				
		60°C	70°C	80°C	90°C	100°C

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Effects of Hydrogen Ion (pH) Concentration on Growth

With the exception of heat, hydrogen ion concentration exerts the greatest influence on bacterial growth. As with temperature, each species has its own range of tolerance. Those pH values above and below the range of the bacterium result in the inactivation of enzymes. While, as in the case of *Enterobacter aerogenes*, high acidity stimulates or induces the production of enzymes which tend to reduce the concentration of acids, there are limits of tolerance for all microorganisms.

Materials:

— Broth cultures of:
  Escherichia coli
  Alcaligenes viscolactis
  Staphylococcus aureus
  Enterobacter aerogenes
— Four (4) glucose agar deeps (pH 3, 5, 7, and 9) with phenol red
— Four (4) Petri plates

Methods:

1. Work in teams of four (4) members, each member testing all the organisms at one pH value.
2. Divide the plates into four (4) sections and label each section with the name (initials) of one of the test organisms. Indicate the pH of the test medium.
3. Melt the glucose agar deeps and pour the plates.
4. Inoculate each of the organisms into the appropriate section by using a single streak of the loop.
5. Incubate at 21°C.
6. Examine for growth at 2 and 7 days and record your team results.

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Effects of Osmotic Pressure Imbalance

The osmotic balance of water entering and leaving the bacterial cell is an important aspect of the growth and survival of bacteria. While some microorganisms tolerate high solute concentrations, as seen in some marine forms, most require an osmotic pressure just slightly lower than that of the cytoplasm.

Generally, and within limits, bacteria are able to survive in **hypotonic** solutions with solute concentrations considerably lower than that within the bacterial cytoplasm as the rigid cell wall tends to limit the amount of water that can enter the cell. However, when bacteria are placed in **hypertonic** solutions, growth may be seriously limited. Hypertonic solutions withdraw water from the cell causing the cytoplasm to become dehydrated and shrink away from the cell wall. Some bacteria are simply inhibited under these conditions and will return to normal when placed in an **isotonic** solution. Others may be irreversibly affected due to the permanent inactivation of enzymes.

The effect of osmotic pressure is of practical importance in food preservation. Such foods as jam, sauerkraut, condensed milk, salted meats and fish, owe their lasting qualities to desiccation of contaminating microorganisms by high osmotic pressure. Preservation does not mean foods are sterile. Many viable forms will grow when the osmotic pressure is more favorable.

Materials:

— Broth cultures of:
  Staphlococcus aureus
  Streptococcus liquefaciens
  Saccharomyces cervisiae or *Halobacterium salinariura*
  Aspergillus niger
— Raisins and raw hamburger
— One (1) nutrient agar deep
— Four (4) nutrient agar deeps with varying concentrations of NaCl (5, 10, 15, and 25%)
— Three (3) nutrient broths with varying concentrations of NaCl (5, 15, and 25%)
— Three (3) malt-extract broths with varying amounts of sucrose (15, 30, and 60%)
— Five (5) Petri plates

Methods:

1. Work in teams of four (4) members.
2. Label all the media as it is picked up from the media cart.
3. Divide the Petri plates into four sections each and label the sections with the name of one of the test organisms. Indicate the NaCl concentrations.
4. Melt and pour each of the plates.
5. Inoculate a single streak within each section with the appropriate organism.
6. Place a small piece of hamburger in each of the tubes of nutrient broth with NaCl. Label appropriately.
7. Place one raisin in each of the tubes of malt-extract broth with sugar. Label appropriately.
8. Incubate at 21°C and observe for 2 to 7 days.

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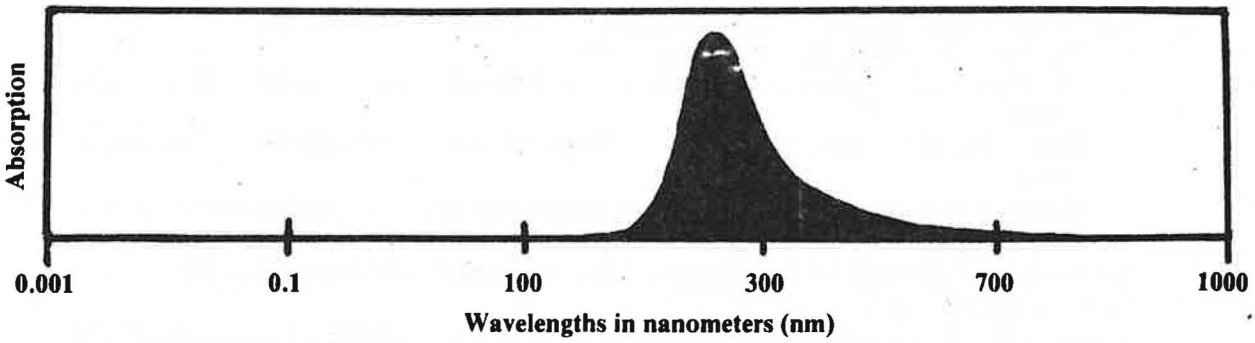
Effects of Ultraviolet Radiation

Short wavelengths of light are lethal and mutagenic to most microorganisms. This includes both the ionizing radiations of gamma and X-rays and the somewhat longer electromagnetic radiations of ultraviolet (U.V.) light. Because of its low penetration, U.V. light has very limited use for sterilization. Its primary value is in surface and air disinfection. The bactericidal effects of U.V. light are limited to a narrow portion of the U.V. spectrum where microbial nucleic acids, primarily DNA, exhibit the strongest absorption.

Bactericidal activity of U.V. light: Absorption by nucleic acids



U.V. light with wavelengths of 265 nm affect the chromosomal DNA of microorganisms by forming covalent bonds between adjacent thymine residues. These bonds distort the molecule and interfere with DNA transcription during protein synthesis and replication during cell division. Sublethal radiations can cause inheritable mutations by altering nucleotide sequences.

The bactericidal effect of exposure of a culture to U.V. radiations can be reduced somewhat by the immediate exposure to visible light with wavelengths between 365—450 nm. This reversal, called **photoreactivation**, results when visible light activates an enzyme, present in some microorganisms, that breaks the bonds caused by U.V. light, allowing the DNA molecule to resume its function.

Materials:

— Broth cultures of:
  Bacillus megaterium
  Staphylococcus aureus
  Serratia marcescens
  Pseudomonas fluorescens
— Four (4) nutrient agar deeps
— Four (4) Petri plates

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Evaluation of Disinfectants and Antiseptics and the Bacteriostatic Action of Chemical Agents

A widely-used method for evaluating the effectiveness of disinfectants and antispetics is the disk test. In this test, paper disks, saturated with various disinfectants, are placed on heavily inoculated agar plates. Following incubation, the plates are examined for zones of inhibition of bacterial growth around the disks. The presence of inhibition indicates the sensitivity of the bacterium to the disinfectant under the test conditions, rather than the degree of effectiveness of the chemical agent. Samples of the medium in the zones of inhibition are tested to determine bacteriostatic activity as opposed to bactericidal action.

Materials:

- Broth cultures of:
  - *Excherichia coli*
  - *Bacillus cereus*
  - *Staphylococcus aureus*
  - *Mycobacterium smegmatis*
- Four (4) nutrient agar deeps
- Four (4) Petri plates
- Samples of disinfectants and antiseptics
- Four (4) sterile swabs
- Sterile disks
- Forceps
- 70% alcohol

Methods:

1. Work in teams of four (4) members using one prepared culture per member.
2. Prepare and dry an agar plate.
3. Inoculate the agar plate with your culture using the swab method for confluent growth. Allow it to dry for fifteen (15) minutes.
4. Place the agar plate over the sensitivity disk pattern. The pattern indicates the proper placement of the disks as described by standard methods.
5. Aspetically dip the edge of a paper disk into one of the chemical agents and allow absorption to moisten the disk. Place the disk on the surface of the agar over one of the dots on the pattern.
6. Repeat the above process using the other chemical agents.
7. Incubate at 37°C until next laboratory.
8. Examine the plates for clear areas around the test disks.
9. Remove a small piece of agar from one of the zones of inhibition with a sterile loop. Inoculate this into a tube of nutrient broth. Repeat, placing another small piece of agar from an inhibition zone into a tube of broth containing a neutralizer for the disinfectant.
   The neutralizers are:
   1. For Zephiran and Roccal ..... 0.5% Naphuride in a nutrient broth
   2. For Virac ..... thioglycollate broth
   3. For most others ..... 0.1% gelatin in nutrient broth
10. Incubate these tubes at 37°C for 48 hours.
11. Examine the tubes for growth.

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Effects of Dyes and Metals

Many of the dyes not only stain bacteria but inhibit their growth in high dilutions. The basic dyes, crystal violet, gentian violet, and brilliant green, have a selective action against Gram-positive bacteria. Unfortunately, the dyes are readily absorbed and neutralized by serums and other proteins, thus limiting their use to selective agents in culture media and to treatment of local lesions on the skin, in the mouth, and vagina.

Numerous metals, such as silver, mercury, arsenic, copper, and lead, exert an oligodynamic effect on bacteria by poisoning enzyme activity. For example, organic compounds of mercury such as merthiolate, Metaphen, and mercurochrome, are relatively non-irritating and have been used as antiseptics for skin and mucous membranes. Metaphan has been found particularly effective against Gram-positive cocci. Studies suggest that mercurochrome is not as effective in vivo as in vitro experiments indicate.

This experiment will demonstrate the use of dyes and metals in the control of bacteria.

Materials:

- Broth cultures of:
  - *Bacillus cereus*
  - *Enterobacter aerogenes*
  - *Staphylococcus aureus*
- One (1) MacConkey agar deep
- Four (4) nutrient agar deeps
- Sample of various metals
- Four (4) Petri plates
- Spatula
- Forceps
- 70% alcohol

Methods:

Work in teams of four members. One member should prepare the study on dyes, while the others test the effects of metals on the different types of bacteria.

Dyes:

1. Melt one (1) MacConkey and one (1) nutrient agar deep.
2. Pour the MacConkey agar into a plate and allow it to solidify.
3. Sterilize the spatula by dipping it into the alcohol and igniting.
4. Aseptically, cut the solidified agar in the plate in half and remove one side.
5. Using the melted nutrient agar deep, pour just enough into the empty half of the plate to fill even with the surface of the MacConkey agar. Allow it to solidify.
6. Inoculate the plate by taking one loopful of each prepared culture and making one single streak across the plate at right angles to the cut, leaving about 2-3 cm between the streaks.

Metals:

1. Melt a nutrient agar deep and cool to 45°C in the water bath.
2. Sterilize the forceps by dipping into the alcohol and igniting.
3. Using the forceps, place samples of different metals on the bottom of a sterile Petri plate, equal distance apart.
4. Using one of the prepared cultures, prepare a pour plate being careful not to disturb the well-spaced metal samples.
5. Allow the agar plate to solidify.
6. Incubate all culture plates at 37°C for 24 hours and then transfer to 21°C until the next laboratory.
7. Examine the plates for evidence of inhibition.

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Interference of Disinfectant Activity

The presence of organic matter or other materials such as lubricants, soaps, and detergents, may inactivate many disinfectants and cause substances that typically show high activity to become inert. There are a number of ways in which these substances may alter the disinfectant activity: (1) surface adsorption by protein colloids, (2) formation of inert or less active compounds, and (3) binding of the disinfectant by active groups of foreign protein.

In this experiment, inactivated yeast cells will represent the interfering organic matter. Several disinfectants will be tested against several representative types of bacteria in the presence and/or absence of organic matter.

Materials:

— Broth cultures of:
  Bacillus subtilis
  Escherichia coli
  Staphylococcus aureus
  Mycobacteria smegmatis
  Inactivated yeast cells
— One (1) tube sterile physiological saline
— One (1) disinfectant (10 ml)
— Eight (8) nutrient agar deeps
— Eight (8) Petri plates
— Two (2) sterile blanks
— Two (2) pipets (1 ml)

Methods:

1. Work in teams of four (4) members with each testing one of the prepared bacterial cultures.
2. Label one Petri plate **Control** and another **Experimental**. Divide the plates into four sections each. Label the sections **1 min.**, **5 min.**, **10 min.**, and **20 min.**
3. Melt the agar deeps and pour the plates.
4. Label two sterile blanks **Control** and **Experimental**.
5. Place 1 ml of sterile physiological saline in the Control tube and then transfer 1 ml of the inactivated yeast to the Experimental tube. To conserve pipets, let one person make these transfers. Transfer the saline first and then the yeasts.
6. Transfer 1 ml of your test culture to each of the two tubes.
7. Note the time **exactly** as you transfer 1 ml of the disinfectant to your Experimental tube. Mix the contents by rolling the tube in your hands. Do not start the Control tube at this time.
8. Remove a loaded loop (with the film intact) at 1, 5, 10, and 20 minutes and streak each sample into the appropriate section of the experimental plate.
9. Repeat steps 7 and 8 using the Control tube. You may want to start this tube after you have taken the 5 minute sample from the Experimental tube.
10. Incubate at 37°C until the next laboratory session.
11. Record the results in a chart, using the following symbols:
    0 = no growth
    1 = light growth (15 colonies or less)
    2 = reduced growth (more than 15 colonies but less than 30)
    3 = heavy growth (individual colonies indistinguishable or too numerous to count)

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Determination of the Effectiveness of Mouth Washes

An effective mouth wash should possess the characteristics of an ideal disinfectant: it should be highly toxic to microorganisms; non-toxic to human tissue; stable at room temperature and in air; have a pleasant odor and taste, and be inexpensive. As no disinfectant possesses all these properties, it is necessary to prepare a disinfectant for this particular use. This experiment is designed to test the relative effectiveness of various commercial mouth washes to sterile water.

Materials:

— 50 ml commercial mouth wash
— Six (6) nutrient agar deeps
— Six (6) Petri plates
— Six (6) sterile water blanks (9.9 ml)
— Three (3) sterile blanks
— Three (3) pipets (1 ml)
— One (1) paper cup

Methods:

1. Work in teams of two members. One team will use sterile water throughout the test and function as a **control** for the other teams which will test different commercially marketed mouth washes.
2. Melt the nutrient agar deeps and place in a 45° C water bath until ready to make the pour plates.
3. Place 4—5 ml of saliva in a test tube, replace the cap and shake the tube to mix well. Label this tube **pretest control**.
4. Rinse your mouth thoroughly with mouthwash or the sterile water if you are on the control team. Discharge this fluid into the sink.
5. Immediately rinse your mouth thoroughly with tap water and discharge this fluid into the sink.
6. Place 4—5 ml of saliva in another test tube and mix as before. Label this tube **first rinse**.
7. Remove 0.1 ml of saliva from the pretest control tube to a 9.9 ml water blank and mix by rolling the tube between the palms of your hands. This is a 1:100 dilution.
8. Transfer 0.1 ml of the 1:100 dilution to a 9.9 ml water blank and mix as before. This is a 1:10,000 dilution.
9. prepare two (2) pour plates from the 1:10,000 dilution tube using 1 ml of the dilution in one plate and 0.1 ml in the other. These plates have a dilution factor of 1:10,000 and 1:100,000 respectively.
10. Repeat procedures 6 through 8 using the first rinse tube.
11. One hour after rinsing your mouth with the mouthwash, place 4—5 ml of saliva in a test tube and mix well. Label this tube **one hour test**.
12. Repeat procedures 6 through 8 using the one hour test tube.
13. Incubate all plates at 37° C until next laboratory.
14. Count the number of colonies on each plate and calculate the number of organisms per ml of saliva. If the number of colonies decreased with the use of the mouth wash, calculate the percent reduction using the following formula:

$$\frac{\text{Number of bacteria in untreated saliva} - \text{Number in the rinse}}{\text{Number of bacteria in untreated saliva}} \times 100 = \text{percent reduction}$$

Isolation of Antibiotics from Soil

Antibiotic substances are distributed widely throughout nature and play an important role in the regulation of microbial populations in soil, water, sewage, and compost. Several hundred antibiotics have been prepared in purified form but only a few of these have been sufficiently nontoxic to humans to be of any use medically. The majority of antibiotic agents that have been isolated fail to show the selective toxicity toward microorganisms necessary for the treatment of infectious diseases. Still, there remains a need for new and better antibiotics. No antibiotic is effective against all infectious organisms and there remain pathogenic organisms for which no effective antibiotic has been discovered.

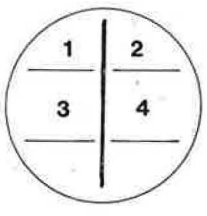
In this experiment, an agar plate will be prepared using a soil sample dilution and a glucose nitrate-salts agar medium. This medium contains glucose as a source of energy, nitrate salts as the only source of nitrogen, and mycostatin to inhibit the growth of molds and is selective for the growth of the genus *Streptomyces* noted for the production of antibiotics. After incubation, those plates with surface growth of *Streptomyces sp.* will be overlaid with nutrient agar seeded with *Staphylococcus aureus* and incubated once more. The presence of clear zones around the *Streptomyces* colonies represents areas where *Staphylococcus aureus* was unable to grow due to the presence of an antibiotic.

Materials:

— Broth culture of *Staphylococcus aureus*
— Soil sample (0.5 gm)
— Three (3) glucose nitrate-salts agar deeps (with mycostatin)
— One (1) nutrient agar deep
— One (1) nutrient agar short (4 ml)
— Four (4) Petri plates
— Sterile dilution blanks
— Pipets (1 ml)

Methods:

1. Work in teams of two (2) members.
2. Melt the glucose nitrate-salts agar deeps and pour three (3) agar plates.
3. Weigh out 0.5 gm of your soil sample and, following your instructor's directions, prepare 1:5000, 1:10,000, and 1:100,000 dilutions.
4. Transfer 1 ml of each dilution to a plate and label the plates with the dilution factor.
5. Using a bent rod, spread the inoculum on the plate.
6. Incubate at 21°C for 2 to 4 days.
7. When good colony development has occurred, add a loopful of *Staphylococcus aureus* to the melted and cooled nutrient agar short. Mix thoroughly and pour over the surface of the *Steptomyces* plate.
8. Incubate at 37°C for 24 to 48 hours.
9. Examine the plate for the appearance of clear zones around some of the colonies.
10. Pick out one of the *Streptomyces* colonies showing inhibition around it and streak it down the center of a glucose yeast extract agar plate.
11. Incubate at 21°C until the growth has become established.
12. To determine the antibiotic spectrum of your new antibiotic, streak four different bacterial cultures at right angles to the line of growth as illustrated below.
13. Incubate at 37°C for 48 hours and determine those organisms most sensitive to the antibiotic.



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Disk Diffusion Test of Antibiotics

Most clinical laboratories now use the Standardized Disk Susceptibility Test to determine the effectiveness of antibiotics against infectious agents.

This procedure provides reliable information not only of the choice of antibiotics but also the most effective concentration to use against a specific pathogenic organism isolated from a clinical specimen. In the test, the zone of inhibition produced by a single antibiotic-containing disk is measured and classified by referring to a standardized table as either resistant, susceptible, or intermediate. In the intermediate zone are those antibiotics to which the bacterium is not fully resistant or susceptible. Though other factors may enter into the choice of an antibiotic, such as possible side effects and patient allergies, this technique offers the physician precise information on which to select the most effective chemotherapeutic agent at the most effective dosage.

STANDARDIZED DISK SUSCEPTIBILITY TEST

DIRECTIONS FOR USE

Quantitative methods that require the measurement of zone sizes give the most precise estimates of antibiotic susceptibilities. The following outline describes such a procedure. Minor variations from this procedure may be used if the resulting procedure is standardized according to the results obtained in the laboratory from adequate studies with control cultures.

A. PREPARATION OF CULTURE MEDIUM AND PLATES

1. Melt previously prepared and sterilized Mueller-Hinton agar medium and cool to 45°–50°C.
2. For the purpose of testing certain fastidious organisms such as streptococci and *Haemophilus* species, 5 percent defibrinated human, horse, or sheep blood may be added to the above medium which is "chocolatized" when indicated.
3. To prepare the plates, pour the melted medium into petri dishes on a level surface to a depth of 4 millimeters.
4. Let the medium harden and allow to stand long enough for excess moisture to evaporate. (For this purpose plates may be placed in an incubator at 35°–37°C. for 15–30 minutes or allowed to stand somewhat longer at room temperature.) There should be no moisture droplets on the surface of the medium or on the petri dish covers. The pH of the solidified medium should be 7.2–7.4. Satisfactory plates may be used immediately or refrigerated. Plates may be used as long as the surface is moist and there is no sign of deterioration.
   Note: Commercially prepared agar plates meeting the above specifications may be used.

B. PREPARATION OF INOCULUM

1. Select four or five similar colonies.
2. Transfer these colonies (obtained by touching the top of each colony in turn with a wire loop) in turn to a test tube containing about 5 milliliters of a suitable liquid medium such as soybean-casein digest broth, U.S.P.
3. Incubate the tube at 35°–37°C. long enough (2 to 8 hours) to produce an organism suspension with moderate cloudiness. At that point the inoculum density of the suspension should be controlled by diluting it, or a portion of it, with sterile saline to obtain a turbidity equivalent to that of a freshly prepared turbidity standard obtained by adding 0.5 milliliter of 1.175 percent barium chloride dihydrate ($BaCl_2 2H_2O$) solution to 99.5 milliliters of 0.36 N (1.0 percent) sulfuric acid. Other suitable methods for standardizing inoculum density may be used; for example, a photometric method. In some cases it may be possible to get an adequate inoculum density in the tube even without incubation.
   Note: Extremes in inoculum density should be avoided. Undiluted overnight broth culture should never be used for streaking plates.

C. INOCULATING THE PLATES

1. Dip a sterile cotton swab on a wooden applicator into the properly diluted inoculum. Remove excess inoculum from the swab by rotating it several times with firm pressure on the inside wall of the test tube above the fluid level.
2. Streak the swab over the entire sterile agar surface of a plate. Streaking successively in three different directions is recommended to obtain an even inoculum.
3. Replace the plate top and allow the inoculum to dry for 3 to 5 minutes.
4. Place the susceptibility disks on the inoculated agar surface and with sterile forceps, or needle tip flamed and cooled between each use, gently press down each disk to insure even contact. Space the disks evenly so that they are no closer than 10 to 15 millimeters to the edge of the petri dish and sufficiently separated from each other to avoid overlapping zones of inhibition. (Spacing may be accomplished by using a disk dispenser or by putting the plate over a pattern to guide the placement of disks.) Within 30 minutes, place the plate in an incubator under aerobic conditions at a constant temperature in the range of 35°–37°C.
5. Read the plate after overnight incubation or, if rapid results are desired, the diameters of the zone of inhibition may be readable after 6 to 8 hours incubation. In the latter case, the results should be confirmed by also reading the results after overnight incubation.
   Note: Microbial growth on the plate should be just or almost confluent. If only isolated colonies are present the inoculum was too light and the test should be repeated.
   Modifications of the inoculation procedure described in 1-3 above, such as the use of the agar overlay method described in Barry, A. L., Garcia, F., and Thrupp, L. D.: "An Improved Single-disk Method for Testing the Antibiotic Susceptibility of Rapidly-growing Pathogens." Amer. J. Clin. Pathol. 53:149–58, 1970, a copy of which is on file with the Office of the Federal Register, may be used if the procedure is standardized to produce results with the control cultures that are equivalent to those obtained with the recommended cotton swab streak method.

D. READING THE PLATES

Measure and record the diameter of each zone (including the diameter of the disk) to the closest millimeter, reading to the point of complete inhibition as judged by the unaided eye. Preferably, read from the underside of the plate without removing the cover, using a ruler, calipers, transparent plastic gauge, or other device. A mechanical zone reader may be used. If blood agar is used, measure the zones from the surface with the cover removed from the plate.

From *Federal Register* 37(191):20527–20529, September 30, 1972.

MICROBIOLOGY OF WATER

The bacteria indigenous to water are not typically of sanitary concern. However, due to the increased numbers of people, of technological development, and the related pollution of water by human and industrial wastes, most water available for municipal use is subject to contamination and may contain pathogenic organisms. Epidemiological evidence has established the relationship between waterborne disease and the presence of organisms of intestinal origin.

The organisms most widely used to indicate fecal contamination are the so-called *coliform group*, which by definition includes all the aerobic and facultative anaerobic, Gram-positive, nonspore-forming, rod-shaped bacteria that ferment lactose with the formation of gas within 48 hours at 35° C, and includes *Escherichia coli* and a number of closely related bacteria. Because of the almost universal presence of *Escherichia coli* in the human intestinal tract and because of the ease with which it can be identified and counted in a water sample, the presence of this bacteria is used as an indication of fecal pollution of water.

Two of the methods most commonly used to detect the presence of coliforms are the standard plate count and the membrane filter technique. The standard plate count is used to determine the effectiveness of water treatment measures by comparing the bacterial numbers before and after each step of the treatment.

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Most Probable Number Method

The Most Probable Number (MPN) method is a multiple tube fermentation technique that consists of three parts:

1. **Presumptive Test.** Fermentation tubes of lactose broth are inoculated with aliquots of the water sample and incubated at 37°C for 24 to 48 hours. The presence of coliform bacteria is indicated by the presence of acid and gas. The presumptive test also makes it possible to obtain a statistical measure of the number of organisms present in the water sample. The number of coliform bacteria present is based on the number of positive tubes found following incubation of the fermentation tubes as indicated on the **MPN Index Chart**.
2. **Confirmed Test.** EMB agar plates are streaked with an inoculum from the positive presumptive tubes and incubated at 37°C for 24 hours. The production of colonies with a green metallic sheen indicates the presence of *Escherichia coli*.
3. **Completed Test.** An isolated colony is transferred to a nutrient agar plate to perform a Gram stain and to a lactose fermentation tube and incubated at 37°C for 24 to 48 hours. The presence of acid and gas in the fermentation tube and a Gram-negative bacilli upon microscopic examination indicates a positive completed test.

Methods:

Work in teams of four (4) members with each team using a different water sample.

Presumptive Test

1. Melt the EMB and nutrient agar deeps and pour the plates. Set them aside for the confirmed test.
2. Shake the water sample to resuspend all material.
3. Using a 10 ml pipet, transfer 10 ml of the sample to each of the double strength lactose fermentation tubes. Label these tubes **10 ml**.
4. Using a 1 ml pipet, transfer 1 ml of the sample to three of the single strength lactose fermentation tubes. Label these tubes **1 ml**.
5. Using the same 1 ml pipet, transfer 0.1 ml of the sample to three single strength lactose fermentation tubes. Label these **0.1 ml**. The remaining tube will be used for the completed test.
6. Incubate at 37° C for 24 to 48 hours.
7. Examine the fermentation tubes for evidence of acid and gas production indicating a positive presumptive test for coliforms. If coliforms are present, determine the most probable number in vour water sample. A negative presumptive test indicates the water sampled is potable. Continue to the confirmed test if your presumptive test was positive.

Confirmed Test

8. Streak the EMB agar plate for isolation using an inoculum from a positive presumptive tube.
9. Incubate at 37° C for 24 hours.
10. Examine the plate for the presence of colonies showing a green metallic sheen. Atypical colonies may be pink, mucoid colonies with dark centers.

Complete Test

11. **Transfer an isolated colony on the EMB agar plate to the single strength lactose fermentation tube. Using the same colony, streak the nutrient agar plate.**
12. **Incubate at 37° C for 24 to 48 hours.**
13. Examine the fermentation tube for evidence of acid and gas production. If the results are positive, make a Gram stain of one of the isolated colonies on the nutrient agar plate. Examine the slide for the presence of Gram-negative short bacilli. A positive completed test indicates the sampled water is not potable.

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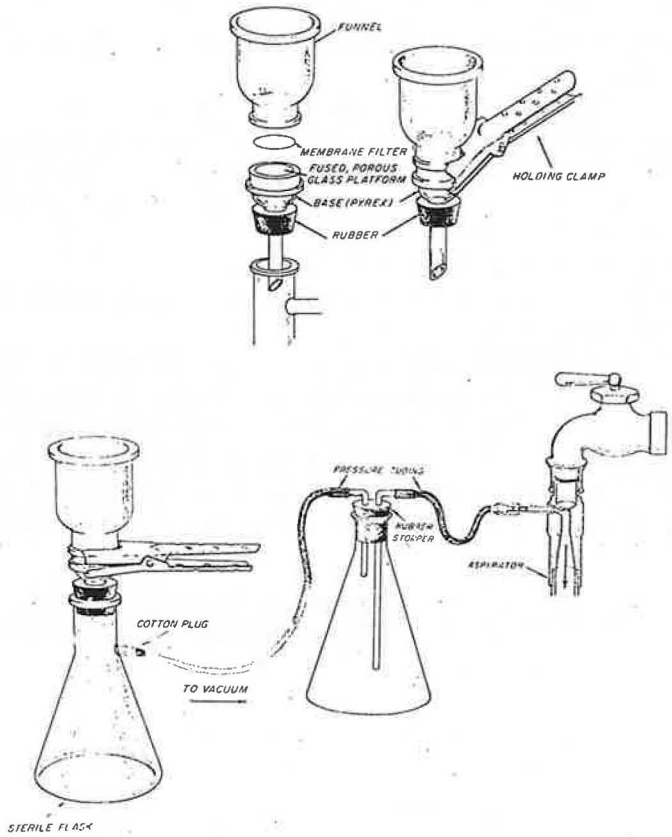
Membrane Filter Technique

The membrane filter technique is routinely used in laboratories to analyze the bacterial load of water and other materials. In this technique, a sample or a dilution of a sample is filtered through a bacteriological filter with a pore size of 0.45 um. Bacteria larger than 0.47 um cannot pass through and are trapped on the membrane which is then placed on nutrient media for incubation and growth of colonies. This technique gives accurate counts if the colony count is between 50 and 200 organisms per filter disk. By placing the membrane on selective and differential media, the number of *coliforms* in a water sample may be determined. This technique has been recognized by the U. S. Public Health Service for the detection of *coliforms* in water.

Membrane-Filter Appratus



Materials:

- Water samples
- One (1) endo broth
- One (1) nutrient broth
- Sterile buffer solution (99 ml)
- Sterile absorbent pads
- Four (4) Petri plates (47 mm)
- Membrane filters (0.45 um)
- Forceps
- 70% alcohol
- Pipets (10 ml and 1 ml)

MICROBIOLOGY OF MILK AND FOODS

In nature, milk is transferred directly to the offspring with little or no possibility of contamination through exposure to the environment. Under artificial methods of collecting and handling, bacteria enter milk from practically everything it contacts. If pathogens are among those that enter, the consumer's health is jeopardized.

The control of the numbers and kinds of bacteria in dairy products is of real concern in the prevention of spoilage of milk and milk products, the transmission of infectious diseases, and in the manufacture of diary products. The problems associated with producing good milk and dairy products involve surveillance of the health of the herd and the personnel tending the herd as well as close attention to the details of sanitary plant operation.

Before mandatory federal inspections of food products, numerous diseases were transmitted by foods. However, there still remain problems associated with the transfer of pathogenic microorganisms by food handlers. Also, animals normally contain microorganisms pathogenic to humans. If the sanitation processes are not adequate or if the original microbial load was too high, they can cause illnesses. The methods used to determine the sanitary quality of foods is to measure the content of certain indicator organisms and the bacterial numbers in foods. As in water samples, the presence of *coliforms* indicates contamination with fecal matter. The presence of large numbers of organisms could mean a greater potential for the presence of pathogens.

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Standard Plate Count for Milk

A total count of bacteria in milk, as well as in other foods, is the most reliable indication of the care with which the milk has been produced and handled. However, it does not reveal the presence of specific human pathogens. Protection against these organisms is handled by animal care and inoculations, human sanitary and health practices, and by pasteurization of the milk itself. The discovery of *coliform* bacteria such as *Escherichia coli* and related species always indicates unsanitary conditions in handling and is an index of the potential presence of pathogenic bacteria.

The standard plate count will be used to determine the number of viable bacteria present in various milk samples. The acceptable standards for milk and dairy products have been set by the U.S. Public Health Service and the California Bureau of Milk and Dairy Products which are considerably stricter. Standards for *coliform* counts have also been set. The *coliform* count will be made by inoculating a measured amount of milk samples on an EMB agar plate, counting the number of *Escherichia coli* colonies and calculating the number of *coliforms* per ml. Similar methods will be used to determine bacterial counts in various other foods.

Dairy Product	**Maximum Number per Milliliter or Gram (California)**
Grade A, prior to pasteurization	50,000 (750 *coliforms*)
Grade A, raw	10,000 (10 *coliforms*)
Grade A, pasteurized milk and milk products (except for cultured products like yogurt and buttermilk)	15,000 (10 *coliforms*)
Grade A, pasteurized cultured products	10 *coliforms*)
Ice cream	25,000 (10 *coliforms*)

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Methylene Blue Reduction Test

The reduction test is a simple procedure used to obtain useful but limited knowledge concerning the bacteriological quality of milk samples. Its primary value is as a screening test to survey a large number of samples at one time. In a milk sample tht contains a alarge number of activly metabolizing microorganisms, the concentration of dissolved oxygen is rapidly reduced. Methylene blue loses its color in an anaerobic environment and is reduced. Therefore, the rate at which methylen blue is reduced following its addition to a milk sample indicates the relative quality of the milk.

Quality of Milk	Time Required for Reduction (Loss of Blue Color)
Excellent—Grade A	More than 8 hours
Good—Grade B	6½ to 7½ hours
Fair—Grade C	2½ to 6 hours
Poor—Grade C	Less than 2 hours

Materials

- Raw milk
- Pasteurized milk from vending machines
- Ice cream
- Reconstituted powdered milk
- Five (5) pipets (10 ml)
- One (1) pipet (1 ml)
- Five (5) sterile blanks with caps
- Methylene blue thiocyanate solution

Methods:

1. The class will be divided into two (2) teams, each team performing reduction tests on all the milk samples. The work may be divided into four parts: preparation of the tests and three subsequent checks at two-hour intervals. Select the time for the preparation of the test so that the final check coincides with the regular laboratory period.
2. Transfer 10 ml of each milk sample into labeled, sterile blanks.
3. Add 1 ml of methylene blue thiocyanate solution to each tube and invert the tube three (3) times **Do not shake the tubes.** This will aerate the milk and affect the reaction.
4. Record the time. Check every two (2) hours, noting the time that methylene blue is reduced to colorless form.

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Standard Plate Count for Foods

Though certain microorganisms are necessary in the production of foods such as yogurt, cheese, and pickles, the presence of others is of real concern in the spoilage of foods and as the causative agents of disease. Foods can serve as carriers of pathogenic organisms which cause such diseases as bacillary dysentary and cholera and as media in which pathogens may grow, some producing endotoxins that cause food infections when ingested and others secreting exotoxins that result in food poisoning.

In this experiment, the standard plate count will be used to determine the viable cell count and the presence of *coliform* bacteria, the indicators of fecal contamination.

Materials:

- Various food samples
- Three (3) nutrient agar deeps
- One (1) EMB agar deep
- Five (5) Petri plates
- One (1) sterile 99 ml water blank
- One (1) sterile 180 ml water blank
- Pipets (1 ml)
- Sterile sand or blender

Methods:

1. Work in teams of four (4) members. Each team will use a different food sample.
2. Melt the agar deeps and place the three nutrient agar deeps in the 45° C water bath.
3. Label the plates with the following dilution factors: **1:10, 1:100, 1:1000,** and **1:10,000.**
4. Pour the EMB agar plate using the plate labeled **1:10.**
5. Weigh out 20 grams of the food sample on sterile weighing paper.
6. If you are using the blender, place the weighed food sample and 180 ml of sterile water in the blender and blend for two (2) minutes. If you are using the sterile sand, add the weighed food sample and the sand to the 180 ml water blank and shake for three (3) minutes. These suspensions are 1:10 dilutions.
7. All the food particles to settle.
8. Transfer 1 ml of the 1:10 dilution bottle to the 99 ml water blank and mix. This suspension is a 1:1000 dilution.
9. Transfer another 1 ml of the 1:10 dilution bottle and place it on the EMB agar plate. Spread using a bent-rod.
10. Remove 0.1 ml from the 1:10 dilution bottle and prepare a pour plate using the plate labeled **1:100.**
11. Remove 1 ml from the 1:1000 dilution bottle and prepare a pour plate using the plate labeled **1:1000.**
12. Remove 0.1 ml from the 1:1000 dilution bottle and prepare a pour plate using the plate labeled **1:10,000.**
13. Incubate the plates at 37° C until next laboratory session. Do not invert the plates to incubate.
14. Examine the EMB agar plate to determine the presence of *coliforms.* especially *Escherichia coli.*
15. Count the number of colonies on the plate having 30 to 300 colonies and calculate the number of bacteria per ml in your food sample. The results will be shared with the class.

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Synder Test for Dental Caries Susceptibility

Dental decay is known to be initiated by the action of lactic acid, as well as other acids, which causes decalcification of tooth enamel. *Lactobacillis acidophilus, Streptococcus mutans*, and *Actinomyces odontolyticus*, among others in the oral flora, produce lactic acid from simple sugars. *Streptococcus mutans* produces exoenzymes that split the sucrose into fructose and glucose, forming long, insoluble, glucose polymers (glucan). In the process, fructose is made available for fermentation. The glucan polymers combine to form a network, called **dental plaque**, that holds the bacteria close to the teeth. The lactic acid bacteria ferment the fructose beneath the plaque causing decalcification and the initiation of dental caries.

The Synder test determines the amount of acid produced by oral flora and uses this as a measure of the susceptibility of an individual to dental caries. Saliva specimens are cultured on a medium containing glucose and pH indicator brom cresol green. This indicator is yellow at pH 4.4 and lower. Decalcification of dental enamel occurs in acid environments of pH 4.4 and less.

Assessment of Dental Caries Susceptibility

Caries susceptibility	Hours of Incubation		
	24	48	72
Marked	Positive		
Moderate	Negative	Positive	
Slight	Negative	Negative	Positive
Negative	Negative	Negative	Negative

Materials:

- Saliva specimen
- Two (2) Synder test agar deeps
- One (1) pipet (1 ml)
- One (1) sterile blank (with cap)
- Ice water bath (beaker with ice)

Methods:

1. Melt the Synder agar deeps and cool to 45°C.
2. Collect saliva over a three (3) minute period. Do not swallow.
3. Thoroughly shake the specimen tube and transfer 0.2 ml of saliva into one of the tubes. Mix by rolling the tube in the palms of your hands. Set the tube in the ice water to chill quickly.
4. Repeat step 3, using the other melted agar deep.
5. Incubate at 37°C for 72 hours, checking every 24 hours for evidence of acid production.
6. Using the table above to interpret your results, record your indicated caries susceptibility.

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Comparison of Scrub Techniques

Cleanliness is generally understood to mean **free from soil**. In food preparation and service, and certainly in the medical professions, its importance as a means of controlling the spread of disease is more clearly understood and appreciated. Since the studies by Semmelweis, it has become routine practice to wash hands prior to examining a patient and to do a complete surgical scrub prior to surgery. It requires about seven to eight minutes of washing with soap and water to remove all transient microorganisms. However, resident microorganisms are removed more slowly and are less susceptible than the transient microbes to the action of antiseptics.

Materials:

— One (1) sterile water blank
— Two (2) sterile swabs
— Green soap and hand brush
— Four (4) nutrient agar deeps
— Four (4) Petri plates with watch glasses
— Disinfectant
— Sterile gauze pads

Methods:

1. Work in teams of two members. Designate yourselves **A** and **B**.
2. Melt the agar deeps, remove the Petri plate cover and aseptically pour just enough agar into each watch glass to fill it **nearly** full. Do not allow it to spill over or touch the top edge. Cover the plate and allow the agar to solidify undisturbed.
3. **A:** Moisten a swab in sterile water and rub it over an area on **B**'s forearm just a little larger than the diameter of the watch glass. Carefully remove the watch glass, place it, agar side down, over the swabbed area on **B**'s arm, place a gauze pad over it and tape it down securely. Allow it to remain in place for 15 minutes. Remove it and replace it in the Petri plate. Label the plate: **B-Control**.
4. **B:** Moisten a swab in disinfectant and swab **A**'s forearm. Allow it to dry. Continue as above. Label this plate: **A-Disinfectant**.
5. **B:** When your first test is completed, wash your forearm thoroughly with soap and water for one minute, rinse, strip, and air dry.
6. **A:** Place a watch glass, agar side down, next to the area first tested on **B**'s arm. Place a gauze pad over the watch glass and tape it down securely. Allow it to remain in place for 15 minutes. Remove it and replace it in the Petri plate. Label this plate: **B-1 minute wash**.
7. **A:** When your first test is completed, scrub your forearm with a hand brush, soap, and water for three (3) minutes, rinse, strip, and air dry.
8. **B:** Repeat step 6 by placing a watch glass on **A**'s forearm. Label this plate: **A-3 min. scrub.**
9. **Incubate all four plates at 37°C** until next laboratory session.
10. Examine the plates and evaluate the four methods of cleanliness.

SEROLOGICAL METHODS IN DIAGNOSIS

Since microorganisms are antigenic in nature and foreign to the human body, the body's immune system responds to their presence by producing specific antibodies that may eliminate these cells from the body. It follows that a patient's serum can be reacted with a known microbial antigen to determine, through antigen-antibody reactions, whether or not the patient has been in contact with the microorganism. Animals can be exposed to microbial antigens to stimulate antibody formation and their serum reacted with unknown antigens for identification. These concepts are the basis of serological tests which are an important part of diagnostic examinations.

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Agglutination Reaction

Agglutination tests involve an antigen-antibody reaction between particulate or cellular antigens and serum antibodies. Bacterial flagella, capsules, and cell components are able to stimulate antibody production which will result in the formation of visible aggregates of cells or particles when combined. An example is the hemagglutination of red blood cells.

In this experiment, the agglutination reaction will be used to identify an enteropathogenic bacterium causing acute gastroenteritis in children and will use somatic (O) and flagellar (H) antigens and antisera.

Materials:

- Broth cultures of:
  - *Escherichia coli*
  - *Proteus vulgaris*
  - *Enterobacter aerogenes*
  - *Serratia marcescens*
- Four (4) sterile physiological saline blanks
- Four (4) sterile blanks
- Pipet (1 ml)
- *Bacto-Escherichia coli* OH and O antigens and antisera
- Serological slide
- 70% alcohol

Methods:

Preparation of Heat-Killed Cultures

1. Work in teams of four (4) members, each member preparing one heat-killed culture.
2. Heat-kill a broth culture of a bacterium by placing the tube in a 100° C water bath for ten (10) minutes.
3. Transfer the heat-killed culture to a centrifuge tube and centrifuge the tube for five (5) minutes.
4. Decant the supernate into the original tube and place it in the discards.
5. Suspend the centrifuged cells in 1 ml and transfer to a sterile blank.
6. Label and refrigerate until next laboratory session.

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Precipitin Reaction

Precipitin tests involve reactions between soluble antigens and serum antibodies which form complexes that precipiate out of solution as visible granules. In the ring test, antiserum is introduced into a series of serological test tubes and the antigen carefully layered over it. After incubation of up to four hours, a ring of precipitate forms at the interface of the antigen and antibody in the tube having the optimum concentration.

Materials:

— Physiological saline
— Bovine globulin antiserum
— Bovine serum dilutions of 1:25, 1:50, and 1:75
— Pipets (0.5 ml)
— Four (4) serological test tubes and rack

Methods:

1. Work in teams of two (2) members.
2. Label three (3) serological test tubes according to the antigen dilution: **1:25, 1:50,** and **1:75**. Label the fourth tube as a **saline control**.
3. Using a different pipet for each dilution, transfer 0.3 ml of each of the bovine serum dilutions into the appropriately labeled tube.
4. Transfer 0.3 ml of physiological saline into the control tube.
5. Carefully overlay 0.3 ml of the bovine globulin antiserum into each of the four test tubes.
6. Incubate at 37°C for 30 minutes.
7. Examine the tubes for a ring of precipitate at the interface of the antigen and antibody. Which tube showed optimal antigen:antibody ratio?

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Medical-Legal Applications of Serological Reactions

The specificity of many antigenic proteins and the sensitivity of such serological reactions as the precipitin test make it possible to identify the species source of blood and tissue samples. Serological reactions are commonly used in the detection and identifiction of blood stains and adulterants of meat products.

A. Identification of an Unknown Stain

Before a suspected stain is tested serologically, it is necessary to determine whether it is blood or some non-biological material. Saline extracts of the sample are mixed with glacial acetic acid, hydrogen peroxide, and benzidine. If the appropriate blood or tissue enzymes are present. The peroxide will be broken down. The oxygen thus released oxidizes benzidine to a delicate blue-green color. This is **presumptive** evidence of blood as these enzymes are also present in materials other than blood.

Materials:

- Samples of stained and unstained cloth
- Four (4) test tubes (13 x 75 ml)
- Physiological saline (5 ml per sample)
- Glacial acetic acid (2 ml per sample)
- Benzidine powder
- Hydrogen peroxide, 3% solution (20 drops per sample)

Methods:

Part I: Extraction of Stain Material

1. Thoroughly clean and rinse tubes with distilled water. These tests are very sensitive to extraneous materials.
2. Label two sets of two (2) tubes: **Unknown** and **Control**.
3. Pipet 5 ml of fresh physiological saline into one set of tubes.
4. Place a 2—4 cm^2 sample of the stained cloth (**Unknown**) and the unstained cloth (**Control**) into the appropriately labeled tubes.
5. Place both samples in the refrigerator and allow about 48 hours for extraction.
6. The extracts of the stained and unstained pieces of cloth will be used for the following tests:

Part II: Presumptive Test of Stain Material

1. Pipet 2 ml of glacial acetic acid in the second set of tubes.
2. Using a toothpick as a spatula, add a single scoop of benzidine power to each tube.
3. Add 20 drops of hydrogen peroxide.
4. Without mixing, layer a small portion of the sample extracts into the appropriate tubes.
5. Compare the **unknown** and **Contol** tubes. The presence of a pale green color at the interface of the two reagents is presumptive evidence of blood.
6. Save all positive presumptive extracts for serlogical analysis.
7. Record your preliminary results.

C. Determination of Unknown Human Blood Type

The blood cells of humans can be differentiated into four types depending on the presence or absence of two chemical substances called A and B. These substances, which are called **agglutinogens**, are antigenic in nature and are located on the surface of the red blood cells. Blood cells having only the A substance are type A, blood cells having only the B substance are type B, blood cells having both A and B substances are type AB, and blood cells with neither A nor B are type O.

Landsteiner found that the blood serum of type A individuals contains natural antibodies which cause the blood cells having B agglutinogens to clump together. Likewise the blood serum of a type B person contains an antibody which will agglutinate the blood cells having A agglutinogens. The serum of an O individual will agglutinate cells of types A, B, and AB, but the serum of a type AB person will not agglutinate the cells of any of the other three groups. This reciprocal relationship is summarized in the following table:

Blood Type	Agglutinogen on Cells	Agglutinin in Serum
A	A	anti-B
B	B	anti-A
AB	A and B	neither
O	neither	anti-A and anti-B

Since the human A and B blood antigens are carbohydrate in nature, they are more stable than proteins and can be identified years later providing the sample has remained dry. Saline extracts of an unknown stain are mixed with standard amounts of known antisera, incubated, and retested against known A and B blood cells. If the agglutinating capabilities of the antisera has been inhibited, it is concluded that the sample contains specific A or B substances. Controls with the unstained sample must be run. The absence of A inhibition, especially when B is present, does not mean A was absent since blood types A_2 and A_3 are difficult to detect by this method.

Materials:

- Sample of blood stained cloth previously prepared by students (2 cm^2)
- Sample of unstained cloth (2 cm^2)
- Four (4) test tubes 13 x 75
- 0.5 ml anti-A serum 1:20 dilution per sample
- 0.5 ml anti-B serum 1:20 dilution per sample
- 0.5 ml 2% suspensions of A and B cells per sample
- One (1) agglutination slide

Fungi

Fungi are among the most plentiful forms of life and are found in most environments, usually in the soil. Most are saprophytic and indispensable in the decomposition of plant and animal material. Economically, they are important in the production of acids, alcohol, and antibiotics. Though most are saprophytic, a few are parasitic. Fungi frequently cause plant diseases, but of the thousands of known species, less than 100 are capable of invading man or animals and less than a dozen produce fatal infections. The diseases produced by pathogenic fungi resemble those caused by *Mycobacterium tuberculosis* in that they develop slowly and cause chronic infections. Fungi do not produce toxins but cause hypersensitivity reactions and lesions such as tissue necrosis and abscess formation.

Clinical specimens containing pathogenic fungi are rarely seen. However, with the increased use of transplants and the need for immunosupressive drugs, the number of fungal infections in hospitals is rising. Rapid identification of clinical fungal isolates is necessary in that fungal infections, particularly the systemic type, are difficult to treat and invariably are fatal if not treated promptly.

Fungi are plants that lack chlorophyll, roots, stems, and leaves. The two major groups of fungi are the molds, which are multicellular, and the yeasts, which are unicellular. The individual filaments of molds are called **hyphae** (singular—hypha). If the filament has crosswalls, it is **septate**. A filament without crosswalls is **non-septate**. The mat of branching, intertwined hyphae is known as a **mycelium** and the entire plant is called a **thallus**. The mass of hyphae on and in the medium are **vegetative mycelium** and are involved in the assimilation of food. The hyphae that rise above the thallus are called **aerial mycelium**. The aerial mycelium may be involved in spore formation and therefore are called **fertile mycellium**. Fungi are placed in four (4) classes, **Phycomycetes, Ascomycetes, Deuteromycetes,** and **Basidomycetes**.

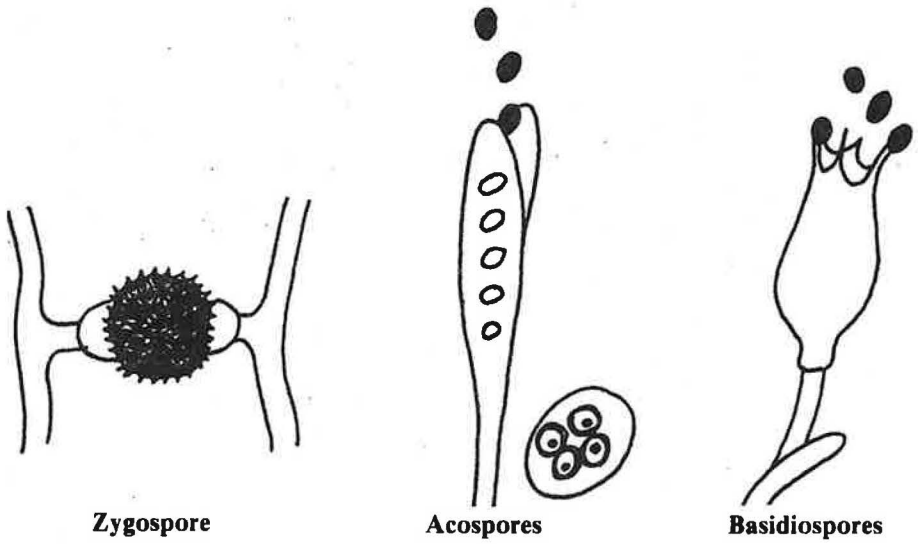
Sexual and asexual spores may be produced. Sexual spores are produced by the fusion of two nuclei from separate plants. Several types are formed:

Acospores. The sexual spores of Ascomycetes are formed within a membrane or sac.

Basidiospores. The sexual spores of Basidiomycetes are formed on a specialized structure, the basidium.

Zygospores. Phycomycetes produce free spores, an example of one is the zygospore of **Rhizopus**. Another example are the oospores of **Saprolegnia**.

Sexual spores of fungi



Zygospore **Acospores** **Basidiospores**

Various types of asexual spores are also formed:

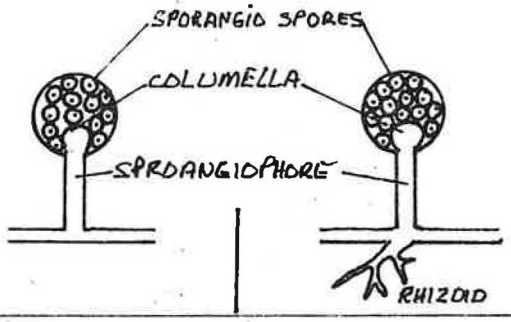
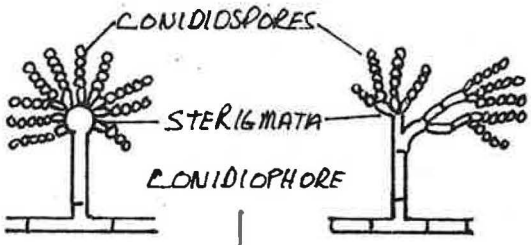
Sporangiospores. In Phycomycetes, the tip of a fertile hypha, a **sproangiophore**, enlarges as many nuclei migrate into the area. A membrane is formed closing the nuclei in the tip now called the **sporangium**. The protoplasm inside the sporangium differentiate around each nucleus forming the sporangiospore. The enlarged section beneath the sporangium is called the **columella**.

Conidiospores. The hyphal cells of septate fungi branch to form **condiophores**. Specialized structures are formed at the tip of the conidiophores and give a characteristic arrangement to the fruiting bodies. Conidiospores are formed from specialized cells called **sterigmata** which are separated individually in loosely attached chains.

Chlamydospores are formed by many fungi when single hyphal cells enlarge and develop thick cell walls.

Arthrospores are formed when single hyphal cells fragment into several spores.

Molds are identified on the basis of the presence or absence of septae, the morphology of the sexual and asexual spores, the groupings of the spores and the pigmentation of the vegetative mycelium, and the spores. Other distinctive structures, such as a root-like **rhizoid** is produced by some. Four genera are included in this study, **Mucor, Rhizopus, Aspergillus,** and **Penicillium.**

Non-septate hyphae **Sporangiospores (asexual)** **Zygospores (sexual)**		**Septate hyphae** **Conidiospores (asexual)** **Ascospre (sexual)**	
No rhizoids	**Rhizoids**	**Black spores**	**Green spores**
Mucor	**Rhizopus**	**Aspergillus**	**Penicillium**
			

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Protozoa

The protozoa are unicellular, eucaryotic, heterotrophic organisms. They have a specialized cell more complex than the bacterial cell and the average cell of a multicellular organism. They have organelles to carry out such functions as nutrition, locomotion, respiration, excretion, and attachment. Most are microscopic or just barely visible to the unaided eye. As a rule, those that are parasitic are smaller than those that are not. The protozoa are primarily asexual, reproducing by binary fission, though some groups reproduce sexually. When subjected to adverse conditions, some strains form resistant cysts. These are important in the transmission of protozoan infections. When suitable conditions for growth recur, the cyst takes in water and the protozoan becomes active once more. All protozoa possess some type of motility during some part of their life. Their classification is based on their means of locomotion, morphology, mode of reproduction, and type of nutrition. However, the means of locomotion is the major criterion. The four classes (or subphyla) of protozoa contain important parasites of man.

Protozoa and Human Disease

Classification	Characteristics	Causative agent (Parasite)	Agent of transmission (vector)	Disease
Sarcodina	Locomotion and capture of food by pseudopodia; most free living; many parasitic; one genera pathogenic to humans	*Entamoeba histolytica*	Person-to-person contact; infected drinking water	Amebic dysentery
Masticophera	Locomotion by flagella, some with undulating membrane; some have chlorophyll and are photosynthetic; some pathogenic to humans.	*Giardia lamblia*	Person-to-person contact; infected drinking water	Giardiasis
		Leishmania donovani	Biting flies	Leishmaniasis
		Trichomonas vaginalis	Person-to-person contact	Trichomoniasis (vaginitis)
		Trypanosoma gambiense	Tsetse fly (*Glossina*)	African trypanosomiasis (African sleeping sickness)
		Trypanosoma cruzi	Reduviid bug (*Triatoma*)	American trypanosomiasis (Chaga's disease)
Ciliata	Locomotion and food capture by cilia; sexual reproduction by conjugation; asexual reproduction by binary fission; some parasitic to humans.	*Balantidium coli*	Person-to-person contact; infected sewage, or drinking water	Balantidiasis
Sporozoa	Adults often nonmotile; many require succession of different hosts in life cycles; nourishment is absorbed directly from host; typically form spores at some stage of life cycle; relatively small; all are parasitic.	*Plasmodium vivax* *P. malariae* *P. falciparum*	Female *Anopheles* mosquito	Malaria
		Toxoplasma gondii	Ingestion of contaminated food	Toxoplasmosis

Helminthes

Collectively, the parasitic worms are called *Helminthes*. The species of medical and public health concern belong to two phyla: *Platyhelminthes* or flatworms which include the two classes of *Trematoda* (flukes) and *Cestoda* (tapeworms) and *Nematoda* or round worms which also includes two classes, the *Nematoda* (hookworms and pinworms) and *Annelida* (leeches). Most parasitic worms have complex life cycles involving one or more intermediate hosts before the definitive host is reached. As many as 30% of the population of the United States are infected by parasitic worms, but in most cases remain asymptomatic. In underdeveloped countries or where sanitary conditions are poor or lacking, large numbers of people have overt symptoms. Laboratory diagnosis relies on the isolation and identification of either eggs or larvae from the infected individual.

Helminthes and Human Disease

Classification	Characteristics	Causative agent (Parasite)	Agent of transmission (vector)	Disease
Platyhelminthes: Cestoda	**Flatworms** **Tapeworms:** Consists of chains of units made up of the scolex with hooks and suckers, neck which is a budding zone and a series of proglottids with both male and female reproductive organs. Have a simple nervous system, excretory system and no digestive system. The intestinal cestodes live as adults in man, while the tissue cestodes in the larval stage.	*Taenia solium:* (pork tapeworm) *Taenia saginata (beef tapeworm)*	Infected meat or fish	Taeniasis
		Clonorchis sinensis	Infected fish	Clonorchiasis
Trematoda:	Flukes: Usually flat, elongated, leaf-shaped. Have an oral and ventral sucker (acetabulum), an excretory pore at the posterior end and a genital pore near the anterior border of the ventral sucker, a blind intestinal tract, an excretory system, primitive nervous system, prominent male and female reproductive system.	*Fasciola hepatica*	Ingested with contaminated drinking water	fascioliasis
		Schistosoma mansoni Schistosoma japonicum Schistosoma haematobium	Cecariae ingested with contaminated drinking water or skin penetration.	Schistosomiasis
Nematoda Nematoda	Roundworms Body cross-section is round. Complete digestive tract, excretory system, nervous system and conspicuous reproductive system, sexes are separate and the males invariably smaller. They go through series in the life cycle which includes ovum, rhabditiform larva with a large mouth and esophageal bulb in intestinal forms, filariform larva with a closed mouth and slender esophagus and adult.	*Ascaris lumbricoides*	Contaminated food, soil, or air	Ascariasis
		Toxocara canis	Contaminated soil	Ascariasis
		Trichinella spiralis	Ingesting infected pork	Trichinosis
		Necator americanus	Rhabditiform larvae penetrate exposed skin or ingestion of eggs	Hookworm disease
		Enterobius vermicularis	Person-to-person contact or ingestion of eggs.	Enterobiasis (pinworm disease)
		Wuchereria bancrofti	*Culex* mosquito	Filariasis (elephantiasis)

Materials:

— Prepared slides and mounts
— Charts of selected Heliminth life cycles

Methods:

1. Examine the prepared slides and mounts. Complete the chart above indicating the location of each type of Heliminth in the body and the method of diagnosis.
2. Examine charts and references to determine the life cycles of the assigned Heliminths.
3. Make sketches and record your observations in your log book.

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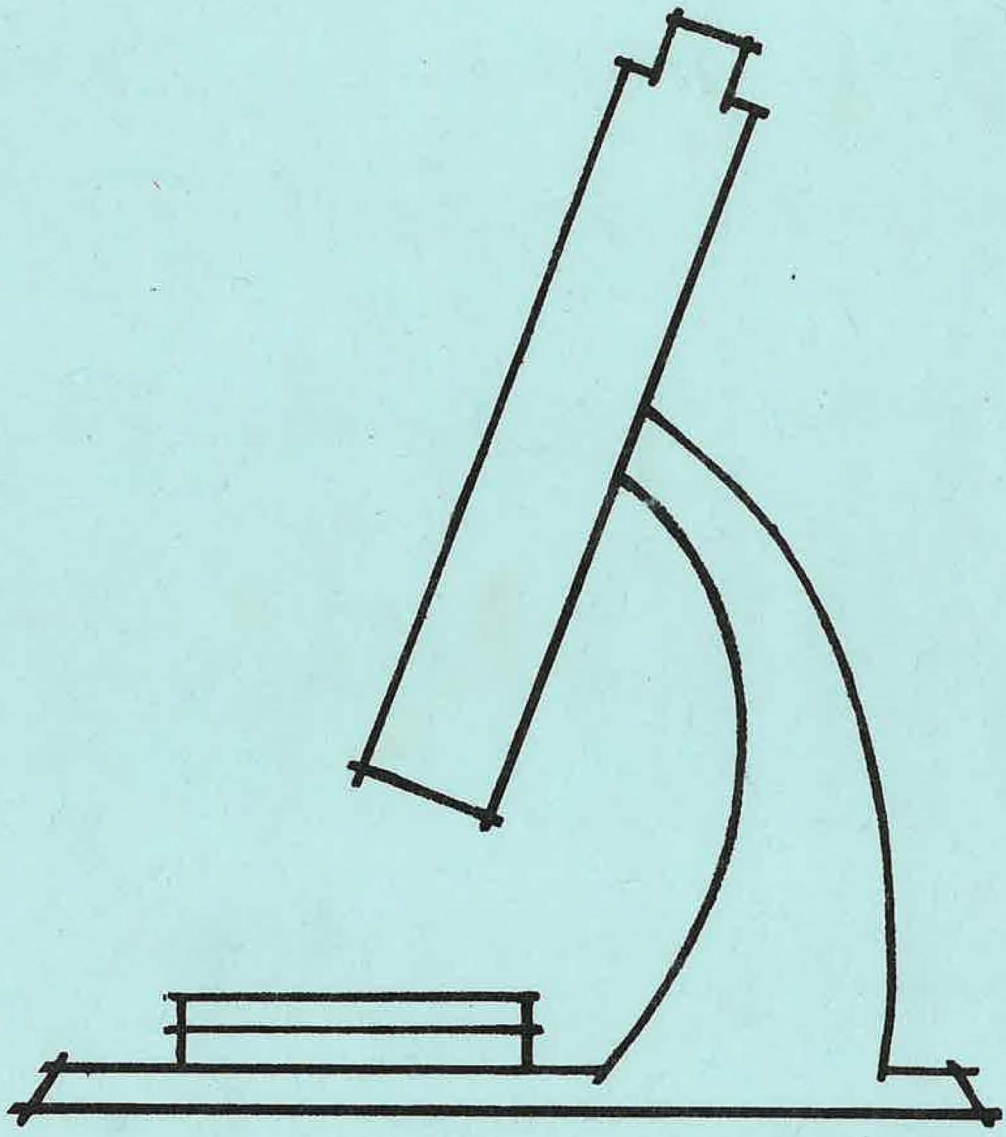
MICROBIOLOGY:
LABORATORY MANUAL



Betty Ward

Take interest, I implore you, in those sacred dwellings which one designates by the expressive term: laboratories. Demand that they be multiplied, that they be adorned. These are the temples of the future—temples of well-being and of happiness. There it is that humanity grows greater, stronger, better.

Louis Pasteur

CONTENTS

UNIT I

UNIT II

UNIT V

Control of Microorganisms

UNIT VI

Applied Microbiology

GENERAL LABORATORY INSTRUCTIONS

The primary purposes of the Microbiology Laboratory are to demonstrate the principles and methodologies of Microbiology and to provide, through experience, the opportunity to develop consistently reliable skills and techniques. The laboratory manual is a guide to introduce the concepts and techniques of microbiological investigations. It leaves you to consider the many variables that present themselves when working with living organisms in the laboratory environment. It is a challenge and a necessary skill to determine why some results differ from the expected. Through such intellectual curiosity, you will learn much more than the original concept on which the investigation was based. You will gain that attitude of analytical perserverance so essential in medical and research sciences. Some procedures may have to be repeated many times before mastery is attained. Do not be satisfied with unsuccessful results due to poor preparation or careless work.

It is essential to study and plan each laboratory investigation before class. Since you will be performing several experiments during the same laboratory period (preparing materials for one investigation, inoculating for another, and collecting the results from still another), it is important to schedule your laboratory work efficiently.

A bound laboratory log book will be kept in which you will record all data and observations. All observations, no matter how seemingly insignificant, should be entered. A useful log book is neat and presents information in a brief, organized, and easily understood manner. Keep your lab book up-to-date, as periodic, unannounced checks will be made.

While the format for log book entries differs from one institution to another, depending on the needs of the researcher or laboratory, the format for keeping your data is as follows:

- **Organization:** Allow one or two full pages for each report depending on the length and complexity of the experiment.
- **Date:** The month, day and year should be listed at the beginning of each experiment.
- **Experiment:** Place the title and number of the experiment at the top of the page beneath the date. You may include the laboratory page number as a useful reference.

RULES AND SAFETY REGULATIONS

Since the microbiology laboratory is a place where microorganisms are cultivated, tested, and stored, accidental contamination of the cultures, yourself, or your friends through carelessness or ignorance of the proper safety measures can be a serious problem. While the organisms used in this laboratory are considered nonpathogenic, it is best to remember that, though the chance is remote, any microorganism given the proper circumstances may become a potential pathogen or be contaminated with a pathogen. Therefore, as a precaution and to establish proper work habits, it is required that the same safety measures used for pathogenic species be applied to nonpathogens.

Your recognition of safety precautions and acceptance of laboratory regulations and procedures are prerequisites to work in the laboratory.

Laboratory Rules:

- A clean lab coat must be worn to protect your clothing and reduce possible contamination of cultures. Your name should on the front pocket.
- Wash your hands thoroughly with soap and water at the beginning and end of each laboratory session and when you leave or enter the laboratory at any other time.
- Clean and disinfect your laboratory desk at the beginning and end of each laboratory period.
- Keep your desk free of non-essential materials at all times. Personal items are to be kept in the areas provided: coats in the closets and books and purses on the shelf beneath your work area.
- Use personal habits appropriate for the laboratory:
  Long hair should be tied back securely.
  Avoid hand-to-mouth operations except as specifically indicated in laboratory exercises.
  Moisten labels with tap water.
  Do not eat, drink, or smoke in the laboratory.
- No unauthorized visitors will be allowed in the laboratory.
- You will be assigned a microscope, a laboratory storage cabinet, and a well-equipped work area. Other equipment and materials will be provided as required. It is your responsibility to properly care for your equipment and experimental materials. Report damaged or missing items to your instructor.

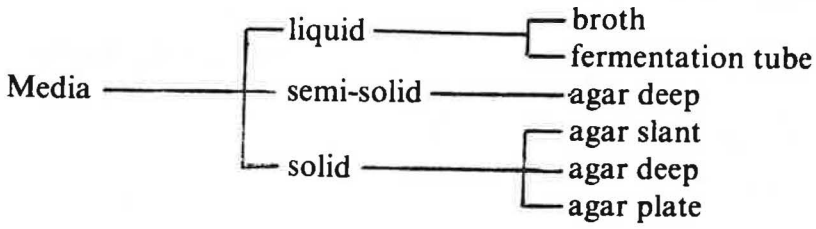
MATERIALS AND EQUIPMENT

CULTURE MEDIA

A variety of culture media, substances that supply nutrients, are used in the laboratory to grow and test the biochemical and physical characteristics of microorganisms. The media may be either liquid, semi-solid, or solid. A liquid medium is called a broth. A broth supplemented with a solidifying agent such as agar is a semi-solid or solid medium, depending on the concentration of the solidifying agent.

Agar, a dehydrated extract of a marine algae, serves as an excellent solidifying agent as it liquifies at 97—100° C and solidifies at 40—42° C, depending on the physical properties of the agar used. Agar has several distinct advantages. Once solidified, it may be incubated over a wide range of temperatures without melting. It provides a solid surface for the growth of discrete colonies, each of which is a cluster of discrete cells that represent clones or a colony of a single species. Also, agar is a complex carbohydrate composed of galactose that is not digested by most microorganisms. Other solidifying agents are gelatin, which is liquid at temperatures above 24° C and digested by many bacteria, and silica gel which is used in cultivation procedures where all organic matter must be excluded.

Media may be placed in test tubes or Petri plates depending on the specific laboratory requirements. Tubed broths provide a simple method for cultivating bacteria and demonstrating certain bacterial characteristics, and chemical reactions. Broth *fermentation tubes*, which contain a small inverted Durham tube to trap any gases produced by microbial metabolism, are used to indicate the anaerobic breakdown of specific sugars. Media supplemented with agar may be tubed, melted, and cooled in either a slanted position, producing an *agar slant* or in an upright position producing an *agar deep*. Agar slants are used to maintain pre-cultures for subculturing and for short term storage. The flat surface area permits the study of colony characteristics as well as certain biochemical reactions. Agar deeps are used to determine a variety of bacterial characteristics such as oxygen relationships and hydrogen sulfide production. They may also be melted and poured into Petri plates. Semi-solid agar deeps are typically used for motility studies.

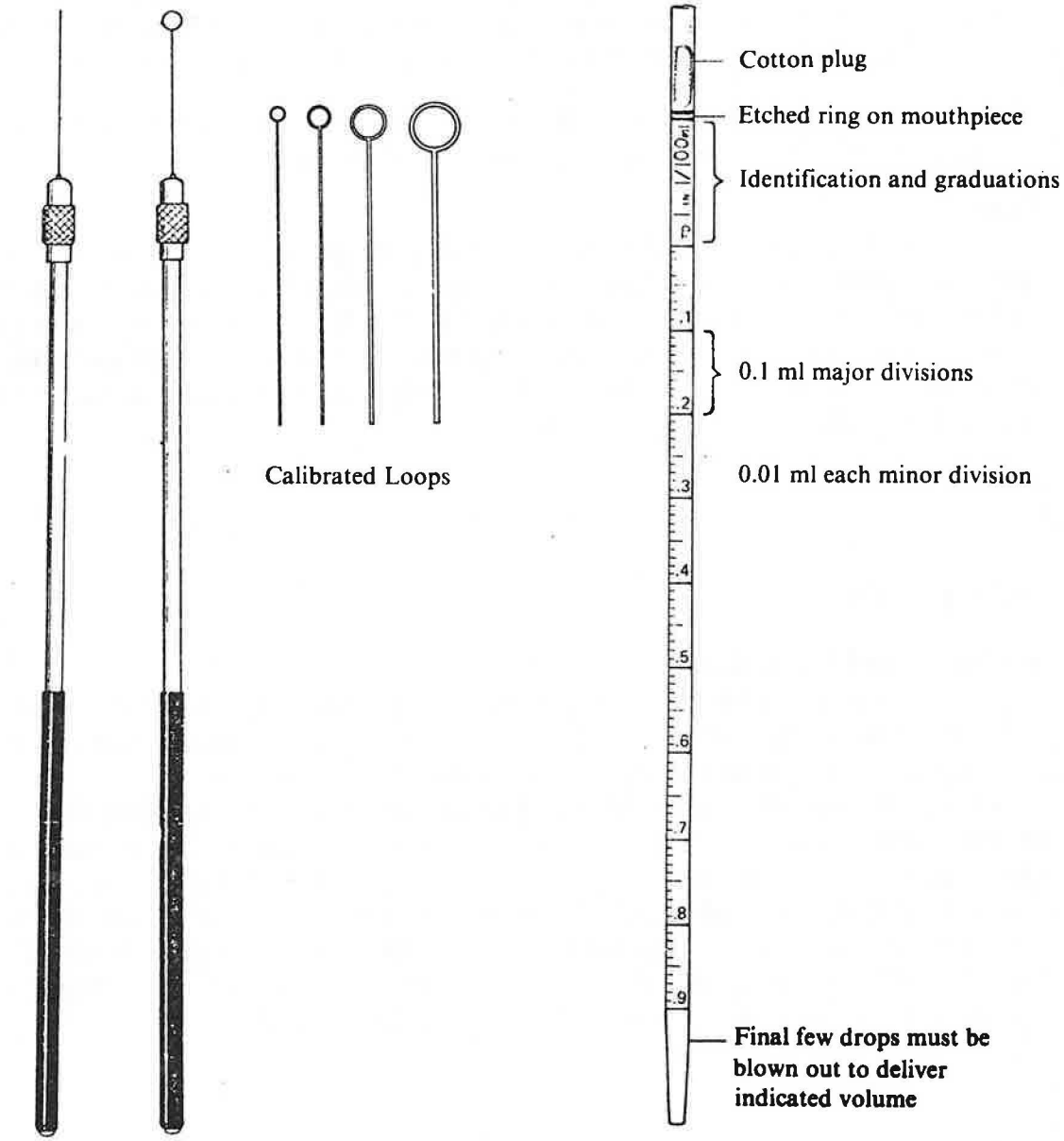


When incubated, the inoculated plates are inverted to prevent the agar from drying out too rapidly. Agar plates provide a greater surface area than agar slants. They are used to isolate microorganisms in the form of colonies, to test bacterial sensitivity to antibiotics and specific chemicals, and to count the number of microorganisms contaminating foods and water, for example.

TRANSFER INSTRUMENTS

Microorganisms must be transferred from one container to another or from a patient to the laboratory for study and maintenance. Such transfers must be made under sterile conditions to prevent possible contaminations. Instruments commonly used in the laboratory for these purposes are illustrated.

Transfer Equipment



STERILIZATION

In the microbiology laboratory, it is mandatory that all media and equipment used to culture microorganisms be sterile, free from all forms of microbial life. Three methods of sterilization are commonly used in laboratories.

Dry Heat

Dry sterilization is achieved in a standard household or laboratory oven and requires that high temperatures be maintained for long periods of time. It is most useful for the sterilization of glassware and other inert objects. A typical sterilization cycle is 160° to 180°C for 1½ to 3 hours.

Moist Heat

The autoclave involves the use of steam under pressure, much like a pressure cooker. It is useful for most types of media, cloth, rubber, and other materials that would be destroyed by dry heat. The standard sterilization cycle is 121°C for 15 minutes at sea level. The length of the cycle will vary somewhat with the amount and type of material being autoclaved.

Filtration

Bacterial filters remove bacteria by the sieve-like action of the minute pores of the filter. They are used to sterilize heat-labile solutions that are destroyed or decomposed by high temperatures and to determine bacterial concentrations in fluids.

The most commonly used filters are the membrane filter and the Seitz filter. The membrane filter apparatus uses a cellulose membrane with a pore size sufficiently small to trap and remove bacteria. The Seitz filter functions on the same principle but uses an asbestos filter disc. Each of these filters are used by attaching them to a suction flask.

1

ENVIRONMENTAL MICROORGANISMS

Take a pinch of soil. Hold it between your thumb and forefinger. You may be holding as many as 200 million bacteria.

Though the number and kinds of microorganisms differ from place to place, depending on environmental conditions, they are abundant and in or on everything. They are numerous in the soil, in the waters and sediments of oceans, lakes, and streams, and on dust particles carried by the air currents. The few exceptions where they cannot exist are in such areas as volcanoes where temperatures exceed the tolerances of even the most specialized microbe, and in healthy, living tissue where the body's natural defenses provide considerable protection from infection. Large numbers of microorganisms are always present on the skin, mucous membranes, and in the intestinal tract of humans and animals. Individuals whose health has been compromised, especially patients in hospitals, are most susceptible to infections. Many harmful bacterial species have become indigenous to the hospital environment and are dangerous sources of infections. Fortunately, most microorganisms are not harmful; in fact, many are beneficial.

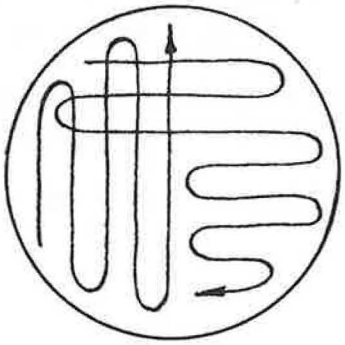
The laboratory, like all other environments, is populated with many microorganisms. This is of particular significance to the microbiologist who must consistently use aseptic techniques to prevent contamination of material from environmental sources.

Because microorganisms are not visible to the naked eye, their presence in any environment must be demonstrated experimentally. This is accomplished by exposing nutrient media to the air or by inoculating the media with material from other sources and observing their growth. The types of microbial colonies that develop may be differentiated from one another by size, shape, color, and consistency. Such characteristics are the result of the environmentally-influenced genetic determinants of each organism. Each well-isolated colony consists of many microorganisms that are the progeny of a single cell. Therefore, each colony represents one microorganism isolated from the environmental source and is counted as such. This experiment will demonstrate the presence, number, and types of microorganisms from different environments and the absolute necessity of aseptic techniques within the Microbiology Laboratory.

Materials

6 prepared agar plates
6 sterile swabs
2 sterile blanks
2 tubes of physiological saline

4. Inoculate the sterile agar plates with a sterile cotton swab in the manner illustrated. The swab streak procedure may be used to isolate individual colonies. At least one-quarter of the surface will demonstrate isolation if the inoculum is not too concentrated. The air, hair, and clothing samples have been inoculated by the open exposure technique and should not be streaked.



Swab Streak Isolation

5. Invert all plates and incubate at the designated temperature until the next laboratory session.

6. Log the inoculations.

7. Discard all contaminated materials and equipment in the designated containers.

Observations

1. Examine the 21°C and 37°C incubated plates.

2. Count the number of colonies on the surface of the plates exposed to air, hair, and clothing.

3. Count the number of colonies in the quarter of the swab-streaked plates that has well-isolated bacterial colonies rather than confluent growth and multiply by four. This will not give an accurate count, but if all streaked plates are counted in the same manner, an estimation of differences in numbers between the various sources can be made.

4. Determine the number of different colony types based on colony size, shape, elevation, color, and texture. Colony types are best evaluated when individual colonies are well isolated from others.

2
ASEPTIC TECHNIQUES

Since microorganisms are everywhere and you are working with a high density of organsims, certain procedures, called **aseptic techniques**, must be followed to prevent contamination during the handling of laboratory cultures. **There is a direct relationship between aseptic technique and pure cultures, successful test results, and the safety of laboratory personnel.** In this experiment, you will aseptically transfer pure cultures of bacteria to liquid, semi-solid, and solid media. The process of transferring cultures from one medium to another is called **subculturing.** The abilty to subculture safely, without contamination, is one of the most essential techniques of the microbiologist. Though the procedure is simple, you will probably be uncomfortable and unsure of your technique. With practice it will become routine and automatic.

GENERAL INFORMATION FOR SUBCULTURING

- The inoculating loop or needle is generally made of 24 or 26 gauge nichrome wire. Under certain circumstances, such as in serological tests, nichrome wire is unsuitable because of its iron content. Platinum wire should be used.
- Generally, an inoculating needle is used to transfer microorganisms from a solid medium and an inoculating loop is used to transfer microorganisms from a liquid medium.
- The inoculating loop or needle must be steilized immediately before and after use. Flame the entire wire to incandescence by first heating the end of the wir nearest the handle. Gradually move the wire through the flame until all parts have been sterilized. Then quickly flame several inches of the handle adjacent to the wire. To avoid spattering or the formation of hazardous microbial aerosols, wet or contaminated wires should be dried over the flame and then flamed as usual.
- Never touch a hot wire to a bacterial culture or specimen or rattle the wire in a tube; a contaminating aerosol will result.
- Once the inoculating instrument is sterilized for use, do not set it on any surface or allow it to touch anything.
- During transfers using tubed materials, remove and hold the caps or cotton plugs between the fingers of the right hand, if right-handed. Never set them down. If one is dropped, replace it immediately with one from a sterile blank.
- Flame sterilize the openings of culture and media tubes bedore and after transferring microorganisms.

4. When the agar has solidified, invert the plate and, using a wax pencil, draw a line across the bottom. Label one side *slant* and the other *broth.*

5. Flame the inoculating loop and allow it to cool. Do not set it down.

6. Hold the slant culture in the left hand and remove the cotton plug with the fingers of the right hand. Flame the mouth of the tube by passing it through the flame several times.

7. While holding the slant nearly parallel with the top of the desk, insert the loop into the tube and carefully touch the surface growth.

8. Reflame the mouth of the tube, replace the cotton plug, and set the tube in the rack.

9. Partially lift one side of the cover of the Petri plate and gently sweep the loop back and forth across the surface of the agar on the side previously labeled *slant.* Replace the cover.

10. Sterilize the loop and place it on the rack.

B. Loop transfer of a broth culture to an agar plate

1. Repeat procedures 5 through 10 given for the loop transfer of a slant culture to an agar plate using a broth culture. **NOTE:** Shake the broth culture to suspend the cells within the media. Do not allow the cotton plug to get wet.

2. Inoculate the surface of the agar labeled *broth.*

3. Sterilize the loop and set it on the rack.

4. Incubate the inoculated agar plate at 21°C until the next laboratory session. Petri plates must be inverted for incubation to avoid disruption of colonies by condensation droplets.

C. Loop transfer of a broth culture to an agar slant

1. Flame the inoculating loop and allow it to cool.

2. Hold the broth culture and agar slant tubes between the fingers of the left hand.

3. Remove the cotton plugs with the fingers of the right hand and insert the loop into the culture broth.

4. Transfer the loop to the agar slant and place the loop at the bottom of the slant and gently draw it up making a wavy line. Do not scratch the surface.

5. Flame the mouths of the tubes and replace the cotton plugs. Set them in the rack.

4. Pick up the agar slant, remove the cotton plug, and flame the mouth of the tube.

5. Place the needle containing the small amount of bacterial growth at the bottom of the slant and draw a single straight line up the surface of the media.

6. Remove the needle, flame the mouth of the tube, and replace the cotton plug. Set the inoculated agar slant in the rack.

7. Flame the inoculating needle and set it on the rack.

8. Incubate at 21° C until the next laboratory period.

F. Needle transfer of a plate culture to an agar deep

1. Repeat procedures 1 through 3 given for the needle transfer of a plate culture to an agar slant.

2. Now, pick up the agar deep, remove the cotton plug and flame the mouth of the tube.

3. Insert the needle into the agar deep by making a stab into the center of the media to within 2 cm. of the bottom of the tube. Remove the needle along the stab entry line. Do not stir the media.

4. Flame the mouth of the tube, replace the cotton plug, and set the tube in the rack.

5. Flame the inoculating needle and set it on the rack.

6. Incubate at 21°C until the next laboratory session.

Observations

- Examine the inoculated media for evidence of growth. If there is any doubt, compare them with tubes of uninoculated media.

- Illustrate the appearance of the growth.

- If the colonies growing on the surface of the agar plates are not well isolated, what steps might be taken next time to correct the problem? Only those colonies that are well-isolated may be assumed to be clonal colonies and may be subcultured for use in biochemical and physical tests to determine their identity.

3

ASEPTIC TRANSFER SKILLS

The success of laboratory experiments and the safety of all concerned is dependent on consistent and effective aseptic techniques. This experiment will test your ability to aseptically transfer media from one tube to another, without contamination, for an extended period.

Materials

Two (2) tubes of nutrient broth
One (1) rubber band

Methods

1. Label both tubes with your name, the number of the experiment, and the date. Use a permanent marking pen.
2. Transfer one loopful of the medium from one tube to the other using aseptic technique.
3. Place a rubber band around your tubes and set them in the designated basket on the front desk.
4. Each day as you enter the laboratory pick up your test tubes. The transfers will be made at the first of each laboratory session.
5. Return your tubes to the basket. They will be incubated until the next laboratory session.
6. Continue this procedure until notified in approximately three weeks.

4

MAINTAINING PURE STOCK CULTURES

In the search for the causative agents of diseases for medical treatment or where the etiology and epidemiology are not known, clinical microbiologists must find and isolate the suspect species from patients or the environment and maintain the microorganism in pure culture. Other microbiologists do not customarily use this search and seizure technique but instead order pure cultures from commercial laboratories that maintain cultures of a wide variety of bacterial species as well as specialized strains of these species. However, the maintenance of pure cultures and the subculture of these cultures for laboratory studies is a necessary skill for the microbiologist.

Materials

Assigned bacterial culture
Recommended transfer and growth medium
Media for the preparation of laboratory experimental subcultues

Method

1. The stock culture of the bacterium must be maintained in pure culture.

2. The bacterium must be transferred regularly to avoid loss of the culture. As the culture ages, nutrients are depleted, metabolic wastes accumulate, and the medium dries.

3. Not all bacteria grow well on nutrient agar slants. Determine and use the appropriate support media.

4. Use your assigned bacterium for each laboratory experiment and record the results carefully. This information will be used, with the information from others, to form an identification key for the **Unknown Cultures** which will be assigned later.

5. Report any problems to your instructor immediately.

6. Prepare one of each of the following cultures of your bacterium:

   Reserve Stock Culture: This culture will not be used except in case of loss or contamination of the **Stock Culture**. This culture must be kept in the refrigerator after being established.

   Stock Culture: This culture will be kept in your storage area and used for preparing **Working Cultures**.

   Working Culture: This culture will be subcultured when required. Check the laboratory schedule and prepare a working culture 24 hours before needed. Some experiments require older cultures. Ask if you are not sure!

5
COMPOUND LIGHT MICROSCOPE

The purpose of the microscope (Greek: micros— small; and scopein— to see) is to magnify otherwise unobservable objects. As such, it is one of the most useful tools of the microbiologist. Virtually all organisms studied in a microbiology laboratory are smaller than 200 micrometers (um) which is the approximate limit of resolution of the naked eye. Bacteria range in size from as small as 0.2 um to 40 um. While biochemical and serological techniques are being used to a greater extent for the identification of microorganisms, certain basic presumptive tests in the identification of microorganisms, require microscopic examination. These studies permit the determination of microbial size, shape, and cell arrangement, capsule and spore formation, motility and staining reactions, among other things.

The student must, therefore, understand the basic principles of brightfield microscopy; be skillful in the operation and use of the microscope, and exercise proper care and maintenance.

Materials

Compound microscope with light source
Prepared slides of:
Paramecium caudatum
Mixed bacterial cell types

A. Care of the Microscope

Each student is assigned a microscope and is responsible for its care and proper use. In that you are sharing your microscope with students from other Microbiology Laboratory classes, it is important you make certain your microscope is clean and in proper working condition. If there are any problems, report them immediately.

Since the microscope is a delicate and expensive precision instrument, the following regulations and procedures must be observed:

- Carry the microscope with the arm of the microscope securely held in one hand and the base supported by the other.
- Place the microscope gently on the desk away from the edge.
- Clean the microscope before and after use. Use only clean, dry *lens paper* to remove dust and oil from the ocular and objectives lenses.
- When you have completed your microscope studies:
  a. Lower the condenser.
  b. Rotate the low-power objective into working position.
  c. Lower the body tube completely.
  d. Replace the dust cover.
  e. Return the instrument to its designated space in the microscope cabinet.
- The microscope cabinet is locked whenever an instructor is not conducting a class. If you wish to use your microscope in the laboratory when classes are not in session, you must sign a microscope check-out slip. You are responsible for the microscope until it is returned to the locked cabinet and your check-out slip has been returned.

Table 1: The Parts of the Microscope

Microscope Part	Function
Ocular or eyepiece	A series of lenses that enlarge the image 10 times (10x).
Ocular thumb wheel	Controls the distance between the oculars so adjustments can be made to fit your eyespan.
Body tube	A long tube which provides the distance for proper focusing of the image.
Revolving nosepiece	A revolving extension of the body tube which carries the objective lenses and can be rotated to change from one objective lens to another.
Objectives	Three (3) cylinders on the nosepiece which contain several lens systems to magnify the specimen: low-power objective (10x); high dry power objective (43—45x); oil immersion objective (97—100x).
Slide holder	Two (2) metal clips, parallel to one another, which hold the slide in position in the mechanical stage.
Mechanical stage	A graduated, moveable device on the stage which is controlled by two knobs. These move the slide back and forth and from side to side. A specimen can be located by recording the coordinates on the graduated scale.
Stage	Supports the mechanical stage and microscope slide.
Diaphragm with lever	A camera-type diaphragm or iris which regulates the amount of light entering the microscope.
Substage condenser	A set of lenses which condense the light waves. Controls the light intensity when raised or lowered.
Light source	A built-in source of illumination which directs the light through the microscope.
Arm	A supporting back frame of the microscope. Provides a handle for transporting the microscope.
Base	A platform which supports the entire microscope.
Coarse adjustment knob	A large round knob which, when rotated, raises and lowers the body tube. Provides approximate focusing.
Fine adjustment knob	A smaller knob which, when rotated, slowly raises and lowers the body tube. Provides precise focusing to sharpen the image of the specimen being observed.

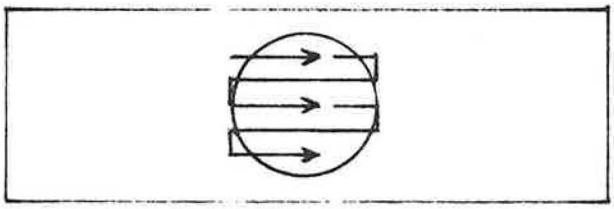
13. Rotate the oil immersion objective into place within the drop of oil.

14. Use the fine adjustment knob to bring the bacteria into sharp focus. If focus cannot be found after a few moments, return to low power objective and relocate the bacteria. Return to the oil immersion objective and focus using only the fine adjustment knob.

    When you have become more adept at working with the microscope, you can use the oil immersion lens immediately without going through the preliminary steps. The procedure is as follows:

    - Adjust the movable body tube so the objective is as far from the stage as possible.
    - Rotate the fine adjustment knob as far as it can be rotated upward.
    - Place a drop of immersion oil on the stained specimen.
    - Looking at the microscope from the side, rack down the oil immersion lens with the coarse adjustment until it touches the oil and continue downward until the objective is just above the level of the slide. Now look through the eyepiece and slowly rotate the fine adjustment away from the specimen. If you have to rotate the fine adjustment more than five or six turns and cannot get the specimen in focus, repeat the procedure, getting the lens as close to the specimen as possible. As long as you first rotate the fine adjustment clockwise, you will not drive the objective lens into the slide during the focusing. This procedure requires a certain amount of technique and experience.

15. To scan the slide move the slide while examining it. Start at the top and move to the side, then down slightly and across again in a systematic search pattern as indicated below:



Slide Scan Pattern

No additional oil is required for this maneuver. Locate the different bacterial cell types. A slight clockwise then counter-clockwise rotation of the fine adjustment knob will give a three dimensional effect to the image.

16. Draw representative cell types in your log book and indicate the magnification used. Label the cells according to their morphology: spherical (coccus; plural, cocci), rod (bacillum; plural, bacilli), and spiral (spirillum; plural spirilla).

17. When you have completed the study, clean the slides and return them to the appropriate slide tray.

18. Thoroughly clean the objective lenses with lens paper.

6

CALIBRATION OF THE MICROSCOPE

The exact measurement of a microorganism is an important aspect of its description. Before such measurements can be made, the microscope must be calibrated. This process involves the determination of the extent of magnification accomplished by each of the objective lens systems and requires the use of two optical devices, an **ocular micrometer** and a **stage micrometer**. The ocular micrometer is a glass disk with parallel, equally spaced but unmeasured lines etched on its surface. It is placed within the ocular tube. The stage micrometer is a glass slide on which a microscopic ruler has been etched. The graduations of the ruler are 0.01 mm apart. It is placed on the stage between the mechanical stage holders.

By superimposing the image of the ocular micrometer over that of the stage micrometer, the distance between the lines of the ocular micrometer can be calculated. This distance is called the **calibration factor**. Because the extent of magnification achieved by each objective lens system differs, each must be calibrated separately. In fact, individual microscopes must be calibrated for each differs, however slightly, from another.

Materials

Ocular micrometer (previously placed within the demonstration microscopes)
Stage micrometer
Prepared slides of mixed bacterial cell types

Methods

1. Clean the stage micrometer with lens paper and place it on the stage between the mechanical stage holders of the demonstration microscope.

2. Focus the microscope using the low power objective.

3. Rotate the ocular until the lines of the ocular micrometer are superimposed over the lines of the stage micrometer.

4. Move the stage micrometer so that one line of the stage micrometer corresponds exactly with one of the lines on the ocular micrometer.

5. Locate the next line on the ocular micrometer that coincides exactly with a line on the stage micrometer.

6. Count the number of ocular and stage micrometer divisions within these two points. The calibration factor for one ocular division is calculated as follows:

$$\text{One ocular micrometer division in mm} = \frac{\text{Number of stage micrometer divisions} \times 0.01 \text{ mm}}{\text{Number of ocular micrometer divisions}}$$

7. Using your numbers, calculate the calibration factor and record the information in your log book for future reference. A suggested format for keeping this data is as follows:

   Microscope number: _____

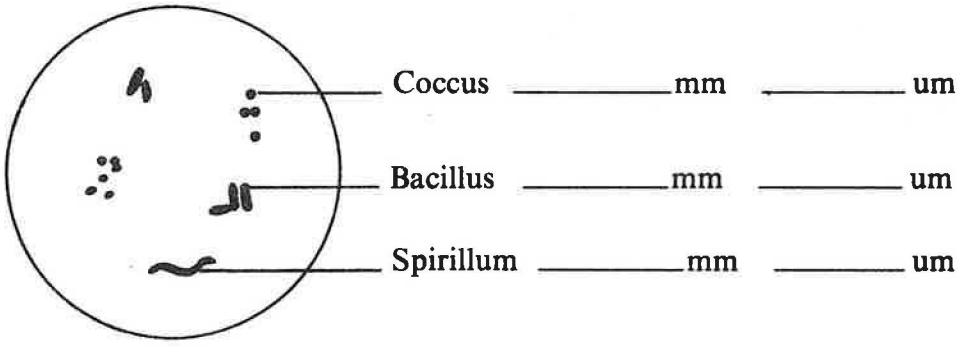
8. Repeat the above procedures using the high power and oil immersion objectives.

9. When you have completed the calibration of the microscope, remove and clean the stage micrometer.

10. Place a prepared slide of mixed bacterial cell types on your microscope stage and center it over the illumination source.

11. Using the oil immersion objective only, determine the size of each of the cell types. You may have to rotate the ocular tube to align the ocular micrometer with the bacteria being measured. Calculate the number of ocular micrometer divisions a cell occupies. The size of the bacteria equals the number of ocular divisions it occupies multipled by the calibration factor for the objective being used. Since some of the microorganisms are not round, both length and width measurements are required.

12. Make a drawing of each cell type. Record size and magnification power used. The following format is suggested:



Prepared slide: Mixed bacterial cell types

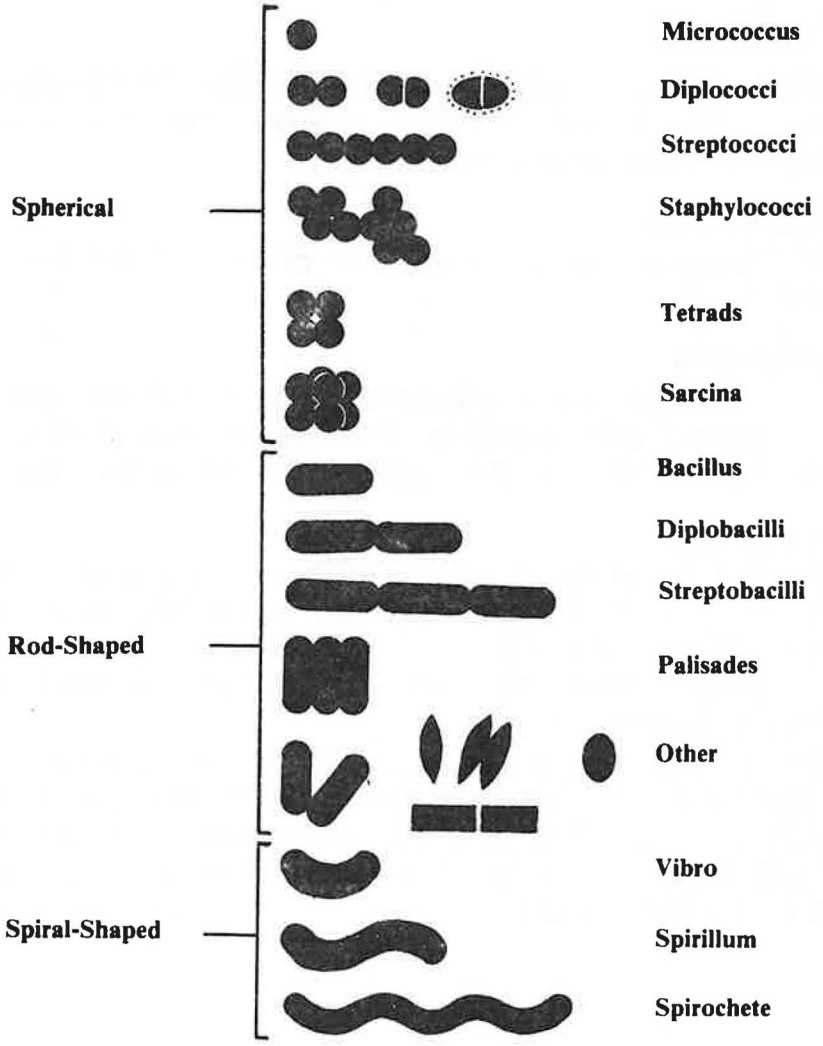
Magnification: _____

BACTERIAL MORPHOLOGY AND STAINING TECHNIQUES

BACTERIAL MORPHOLOGY

The size, shape, and cell arrangement of bacteria is typical of the species and useful in differentiation and classification. Since the size of a bacterial cell varies during growth, this characteristic is not appropriate in the identification of similar groups of organisms. However, most bacteria maintain a relatively constant morphology and cell arrangement (with important exceptions) and may be classified on the basis of these characteristics. There are three basic shapes among bacteria: *spherical* (coccus; plural— cocci), *rod-shaped* (bacillus; plural— bacilli), and *spiral-shaped* (vibrio; spirillum; plural— spirilla, and spirochete). Some bacilli are so small they appear as cocci and are called *coccobacilli* and others are *pleomorphic*, exist in different shapes. Bacteria also assume characteristic cell arrangements based on the way cell division and subsequent cell separation occurs in that particular species.

Basic Shapes and Arrangements of Bacterial Cells



Solid media cultures. One or two loopsful of water should be placed on the slide and the culture added to the water using a sterile inoculating needle. The cells are blended with the water so they cover an area about the size of a dime.

- **Heat Fixation**

  To **fix** a smear, the slide with the smear on the upper surface is passed three or four times through the flame of the Bunsen burner. This kills the bacteria and causes the bacterial proteins to adhere to the slide. A properly fixed smear will not wash off during the staining procedures and will preserve the structures of the cell in their respective forms.

7

SIMPLE STAINING

In the simple stain, the bacterial smear is stained with a single cationic (basic) dye. Since the dye is positively charged, it is attracted to the negatively charged components of the bacterial cell. The purpose of the simple stain is to demonstrate cell morphology and arrangement. Occasionally, endospores may be seen as unstained refractive bodies within bacterial cells.

Materials:

Slant or broth cultures of:
- *Escherichia coli*
- *Staphylococcus aureus*
- *Bacillus subtilis*

Methylene blue
Safranin
Toothpicks

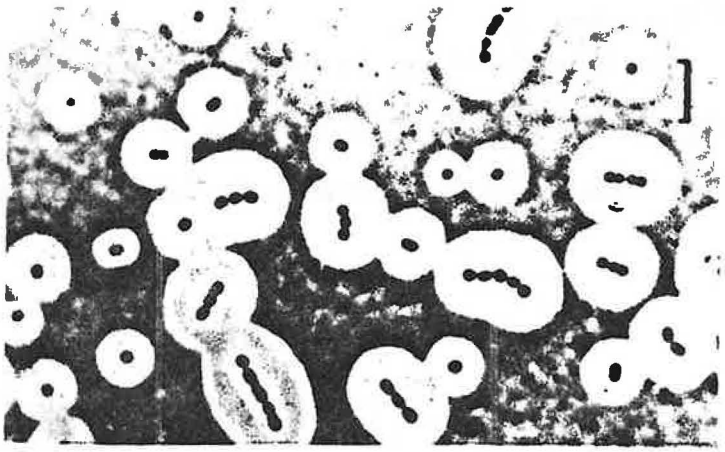
Methods:

1. Divide two slides into two sections each.
2. Remove a small specimen of material from the gum line of one of your rear molars. Using a toothpick, emulsify it with water on one of the sections of a slide, and prepare a smear.
3. Aspetically transfer an inoculum of each of the three (3) cultures to the other sections.
4. Dry thoroughly and heat fix. The drying may be accelerated by holding the slide high above the flame.
5. Stain the slide with the oral specimen with methylene blue for 3—5 minutes and gently wash with tap water to remove the excess stain.
6. Blot the slide dry between a folded sheet of bibulous paper. Do not rub. The stained smear is now ready for observation.
7. Stain the second slide with safranin for thirty (30) seconds and gently wash to remove the excess stain. Blot dry.
8. Examine the prepared slides using oil immersion. The stained oral specimen will contain a few epithelial cells. These cells will appear as large flat cells. The nucleus will be stained blue. The bacterial cell will be much smaller and a darker blue. Compare the morphologies of the three bacterial cultures with those in the oral specimen.
9. Log your results as drawings and discussion.

8

NEGATIVE STAIN

The negative stain technique requires the use of either India ink which is composed of particles too large to enter the cell or a negatively charged, anionic, stain which is repelled by the normally negative charge on the surface of bacteria. In either method the capsule remains unstained and discernible against the darkened background. If the bacterial cell is treated with a contrasting stain, the clear capsular material is more distinct. Since heat fixation is not required, the cells are not distorted and the natural size and shape may be seen.



Photomicrograph of wet mount of an encapsulated bacterium in India ink. The scale line shows the size of 10 um at this magnification.

The ability of bacteria to produce capsules is an environmentally controlled genetic characteristic. Under normal conditions, most bacteria produce a loosely attached slime layer. Only a few bacterial species produce well-developed capsules. Cultures of encapsulated species give a stringy texture to fluid media and form moist, glistening colonies on solid media. Capsules tend to increase the virulence of pathogens by inhibiting phagocytosis. In some pathogens the capsules are antigenic and stimulate the production of antibodies which, in the case of Pneumococci, react with the capsule causing a swollen appearance called the Quellung reaction, a clincally useful diagnostic procedure.

Materials

Tryptose-phosphate slant cultures of:
- *Alcaligenes viscolactis*
- *Flavobacterium capsulatum*
- *Proteus vulgaris*

India ink (freshly filtered)
Loeffler's methylene blue
One (1) small beaker for tap water rinse

9

GRAM'S STAIN

The Gram's staining procedure is indispensable in the identification of unknown organisms. It is routinely the first test performed in the clinical laboratory and provides the initial clues as to the identification of a pathogen. The Gram's stain involves the use of four reagents:

Primary stain. The primary stain, *crystal violet*, functions to color all cells a deep blue.

Mordant. The mordant, *Gram's iodine*, forms a complex with the dye, fixing it to the bacterial cell.

Decolorizer. *Acetone-alcohol* or 95 percent alcohol, releases the stain complex from the cell, decolorizing some cells.

Counterstain. A contrasting stain, *safranin*, replaces the primary stain in those cells unable to retain the primary stain.

When thus stained, bacteria are either blue, Gram-positive, having retained the primary stain, or pink, Gram-negative, having lost the primary stain and been colored by the counterstain. The ability to resist decolorization is related to the chemical make-up and structure of the cell wall. Most cells of living things, including humans, are Gram-negative. It is the Gram-positive characteristic which is distinctive, found only in yeasts, a few molds, and some bacteria. Older cells of some Gram-positive species tend to lose their Gram-positiveness and appear Gram-negative. The organisms on the slide will appear **Gram-variable**, some cells Gram-positive and others Gram-negative.

The Gram's staining reaction correlates with important physiological and cytological traits. Gram-positive cells are more sensitive to antibiotics and dye bacteriostasis and require more complex media for growth, while Gram-negative cells are more susceptible to cell digestion by strong alkali and acids.

In such an important technique one should be aware that variations in the Gram procedure critically influence the results obtained. Such factors as the density of the smear, the concentration of the reagents, the length of time used in washing and decolorization must be considered. Excessive decolorizing may cause Gram-positive organisms to lose the primary stain and appear Gram-negative when counterstained, while insufficient decolorizing may allow Gram-negative organisms to retain the primary stain and appear Gram-positive. The reliability of the results is dependent on the skillful use of precise and consistant Gram's staining procedures.

10
ACID-FAST STAIN

The Acid-fast stain is a differential stain that compares the resistance of cells to decolorization by acids. Most bacteria are easily stained with carbolfuchsin and, when treated with acid-alcohol, decolorize completely. However, it is relatively difficult to stain certain species with carbolfuchsin, however, once stained, they are just as difficult to decolorize, even with acid-alcohol. Those that retain the carbolfuchsin are called *acid-fast* while all other microorganisms, which are easily decolorized by acid-alcohol, are *non-acid-fast*. This is an excellent differential stain and is used clinically in the identification of the tuberculosis bacillus, *Mycobacterium tuberculosis*, the leprosy bacillus, *Mycobacterium leprae*, and certain species of *Nocardia* which causes nocardiosis, a pulmonary disease. It is also used to differentiate a number of harmless acid-fast saprophytes.

The property of acid-fastness is derived from the presence of many complex lipids, fatty acids, and waxes within the cell wall. Acid-fast bacteria have a high cell wall lipid content, approximately 40 to 60 percent. Gram-negative bacteria have no more than 20 percent and Gram-positive bacteria have only 1 to 4 percent. The lipid content is also responsible for the characteristic rough, wrinkled, dry surface associated with colonies of mycobacteria.

Many textbooks, and Bergey's Manual, state that mycobacteria are Gram-positive. However, it is impossible to Gram's stain an acid-fast organism. First, the stain will not penetrate the cell unless heat or chemicals are used. Second such stained cells will not decolorize with Gram's alcohol. Their retention of the primary stain is due to their acid-fast characteristic rather than to any Gram's staining chracteristic. Therefore, acid-fast bacteria are neither Gram-positive nor Gram-negative. The acid-fast stain uses three different reagents:

1. **Primary stain.** The primary stain, *carbolfuchsin*, is soluble in lipoidal material and so will penetrate and be retained by the cell wall. Penetration is also facilitated by the application of heat which drives the carbolfuchsin through the wall and into the cytoplasm.

2. **Decolorizer.** Prior to decolorization, the smear is cooled to allow the waxy cell wall substances to harden. *Acid-alcohol* is applied to decolorize the cell. Acid-fast cells resist decolorization and remain **red.** Non-acid-fast cells are decolorized and transparent.

3. **Counter Stain.** *Methylene blue* is used to stain the decolorized non-acid-fast cells **blue.**

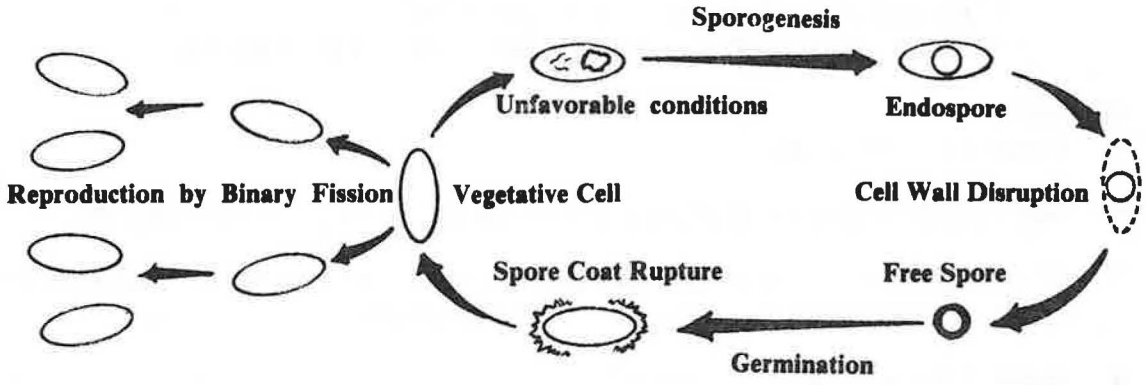
11

SPORE STAIN

Spores represent a chemically and physically resistant form of the vegetative cell of bacteria, algae, and fungi. Due to the composition of the spore coat, the spore is resistant to the harmful effects of excessive heat, desiccation, freezing, radiation, and chemical agents including some of the commonly used microbial stains. Although bacterial cells sporulate under conditions not yet fully understood, spores, nevertheless, are a survival mechanism. When conditions are favorable, the spore will germinate and become a metabolically active and less resistant vegetative once more.

Though bacterial spore formation has been reported in *Sarcina urea* and a *Vibrio* species, it is a distinguishing characteristic of two major genera, *Bacillus* and *Clostridium*. They are associated with such diseases as anthrax, *B. anthracis*, gas gangrene, and food poisoning, *C. perfringens*, botulism, *C. botulinum* and tetanus, *C. tetani*. The number, location, and appearance of spores are used in identification of bacterial genera.

Life cycle of a spore-forming bacterium



Location of bacterial endospores

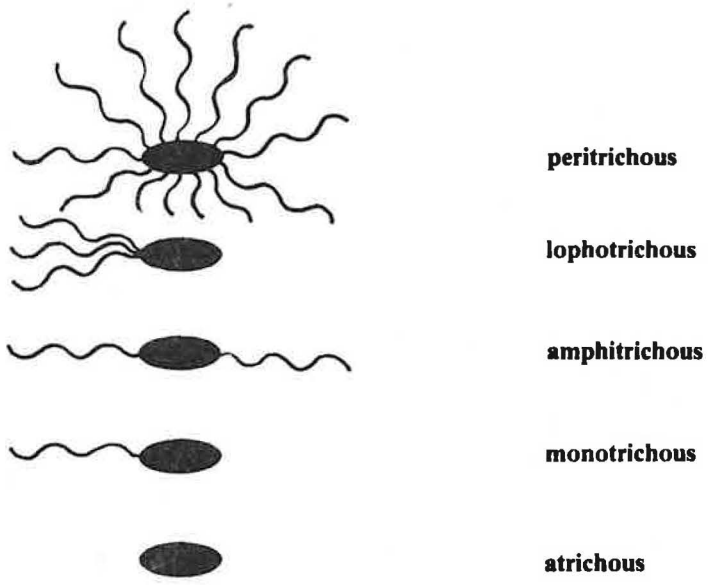
Spores may or may not be swollen

Central **Subterminal** **Terminal**

MOTILITY DETERMINATIONS

Many bacteria are actively motile by means of flagella, long, whip-like appendages that enable bacteria to propel themselves through liquid media. They are present in practically all species of vibros, spirilla, and spirochetes, some bacilli, and an occassional non-pathogenic coccus. They tend to be lost with age. The possession, number, position, and specific antigenic properties of flagella are important cytological characteristics. Bacteria may be classified according to the number and location of their flagella.

Types of Flagella



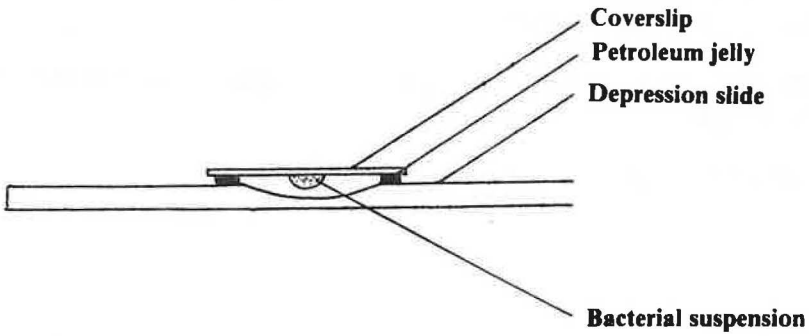
Bacterial flagella have a diameter of 0.01 to 0.03 um and are, therefore, not visible using the light microscope. Special stains that precipitate on the surface of the flagella increase their apparent diameter so they may be seen and their number and position determined. Prepared stained slides of bacterial flagella are available on demonstration microscopes in the laboratory. Flagella are also detected by serological techniques that determine the presence of flagellar antigens. These techniques are not routinely used in the laboratory. The presence of flagella is more readily determined by other procedures such as the wet mount, hanging-drop technique, and motility medium.

Prepared stained slides of bacterial flagella are available on demonstration microscopes in the laboratory. Determine the bacterial flagella classification of the specimens.

12
MOTILITY DETERMINATIONS: HANGING DROP

In the Hanging Drop technique, bacterial motility is observed directly by viewing live bacteria in a liquid medium. Care must be taken to distinguish between true motility and the random motion of brownian movement. An advantage of the hanging drop technique is that it provides an undistorted view of the size, shape, and movement of microorganisms.

Hanging Drop Slide



Materials:

Young cultures of:
- *Bacillus subtilis*
- *Proteus vulgaris*
- *Micrococcus luteus*

Depression slide and cover slips
Petroleum jelly and applicator sticks

Method:

1. Thoroughly clean a depression slide. Using an applicator stick, place a thin layer of petroleum jelly around the edge of the concave well.
2. Using sterile technique, place a loopful of *Bacillus subtilis* in the center of the cover slip.
3. Invert the depression slide and place the concave well over the bacterial suspension on the cover slip.
4. Press down gently to form a seal between the slide and the cover slip.

13
MOTILITY DETERMINATIONS: WET MOUNT

An advantage of the Wet Mount technique is that the slide is easily prepared and, like the hanging drop technique, it permits viewing microorganisms under natural conditions. It requires that care be taken to distinguish true motility, Brownian movement, and the movement caused by water currents which develop in wet mounts due to heat and gravity.

Materials:

Young cultures of:

Bacillus subtilis
Proteus vulgaris
Micrococcus luteus

NOTE: These cultures are also to be used for the hanging drop and motility medium studies.

Methylene blue (1:5000 dilution)
Cover slips

Methods:

1. Transfer four loopsful of *Bacillus subtilis* or *Proteus vulgaris* to a clean microscope slide and add a drop of diluted methylene blue. Place a cover slip over the mixture.

2. Place the slide on the microscope stage and focus using the low power objective. Reduce the light intensity.

3. Examine the preparation using the oil immersion objective.

4. Record your observations. Which of the two methods do you prefer? Would it be helpful to add a loopful of diluted methylene blue to the handing drop slide? Why should young bacterial cultures be used for motility testing?

CAUTION:

Wet mount and hanging drop slide preparations contain living bacteria. Discard these preparations in the suitable container of disinfectant.

14

MOTILITY DETERMINATIONS: MOTILITY MEDIUM

Motility tests are generally performed using a motillity medium instead of the hanging drop or wet mount techniques. A motility medium is a semisolid agar in which the concentration of agar is less than that of conventional agar media. Bacteria are inoculated into the medium using the stab technique. The migration of motile bacteria from the inoculation site can be seen after incubation. Motile bacteria move through the semisolid medium and their growth produces turbidity throughout the tube, whereas non-motile bacteria grow only along the line of the stab inoculation.

Materials:

Young cultures of:

Bacillus subtilis
Proteus vulgaris
Micrococcus luteus

Motility agar deeps (2)

Methods:

1. Using sterile technique, remove an inoculum of your assigned culture with an inoculating needle and inoculate the motility agar by stabbing down the center of the tube, being careful not to touch the bottom. Withdraw the needle along the stab line so as not to spread the inoculum within the medium.

2. Using a toothpick, collect a specimen of tartar from the gum margins of your rear molars. Transfer the specimen to a sterilized inoculating needle and inoculate the other tube of motility medium using the same technique.

3. Incubate both tubes until the next laboratory period. Examine the tubes and those of your partners for the presence or absence of motility. If growth is restricted to the stab line and is well demarcated, with no growth in the rest of the agar, the organism is considered *nonmotile*. Growth spreading through the medium indicates a motile organism.

4. Record all results and conclusions. Do the motility agar studies agree with your observations of the same organisms in the hanging drop and wet mount slides? Which of these three methods do you prefer?

15

CYTOLOGICAL CHARACTERISTICS OF AN UNKNOWN BACTERIUM

Bacterial cytology requires the skillful preparation of bacterial slides and use of the microscope. It is one of the first steps in the identification of bacteria. In this study you will be given an opportunity to demonstrate your skill by determining the cytological characteristics of an unknown bacterial culture. You will have one laboratory period to complete your study. Work independently. This is a test of your mastery of these techniques. You may use your laboratory manual.

Materials:

Unknown bacterium, assigned by number.

Method:

1. Determine the following:
   - Cell morphology
   - Cell arrangement
   - Gram stain reaction
   - Acid-fast stain reaction
   - Motility
   - Endospores
   - Capsules
2. Log your results and have your log book initialed by your instructor.
3. Write a brief report on your study and turn it in next laboratory period.
4. Indicate which of the following organisms may have been the one assigned to you and state the reasons for your conclusions.
   - *Escherichia coli*
   - *Bacillus subtilis*
   - *Staphylococcus aureus*
   - *Streptococcus faecalis*
   - *Mycobacteria phlei*
   - *Sarcina lutea*
   - *Klebsiella pneumoniae*
   - *Proteus vulgaris*

PURE CULTURE TECHNIQUES

In the microbiology laboratory, the testing and identification of microorganisms requires that the microbial specimen being studied be a *pure culture*, a single species of cells free of unwanted or contaminating organisms. Usually many different culture types are associated together in *mixed cultures*. To study a particular microorganism in terms of its special characteristics, the organism must be isolated or removed from the presence of contamination species. In the hospital setting, patient specimens are routinely brought to clinical laboratories for isolation of the infectious agents. Without proper isolation, the identification and antimicrobial susceptibility testing of the infectious agent would not be possible.

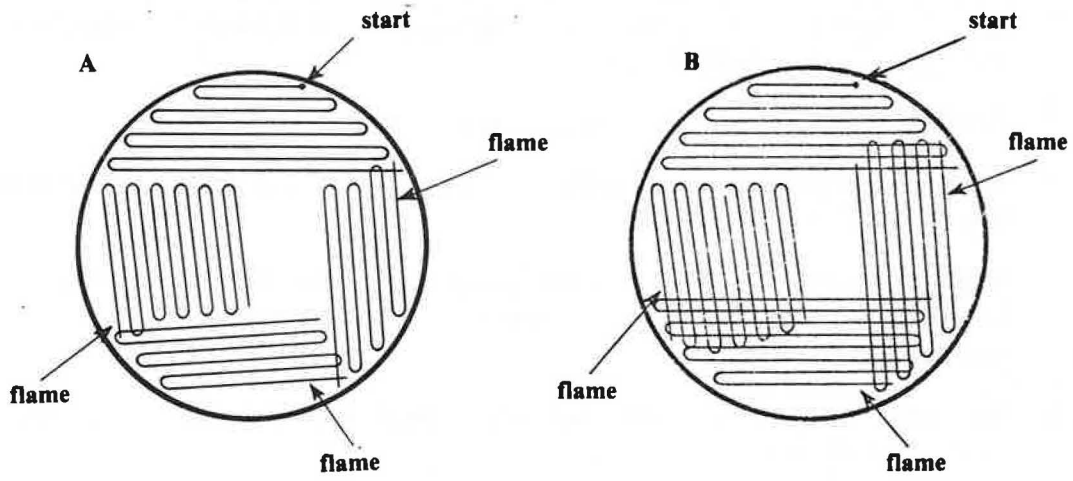
Procedures that permit the isolation of a single species from a mixed culture are **pure culture techniques** and involve not only the mechanical separation of microorganisms as in the streak or spread techniques but the use of various media that impose cultural conditions which permit selection and isolation.

16

STREAK-PLATE

The streak-plate technique provides a simple means of separating mixed cultures. Clinically, it or the swab-streak modification is the method of choice. A loop, containing the inoculum, is streaked onto the surface of an agar plate spreading the individual cells apart. The cells multiply during incubation and form isolated colonies of pure cultures. To be absolutely sure of the purity of the isolated colonies, an inoculum should be transferred to about 2 ml of broth or physiological saline, shaken to disperse the cells and streaked on another agar plate. If the colony was pure, all the resulting colonies will appear the same.

Streak Plate Patterns



Streaking patterns vary with the concentration of the microorganism, the amount of overlap being the significant difference. Pattern A is used when bacterial concentrations are high and B when they are low.

Materials:

Mixed broth cultures of:
- *Escherichia coli*
- *Serratia marcescens*
- *Micrococcus luteus*

(1:1:14 ratio in order listed)

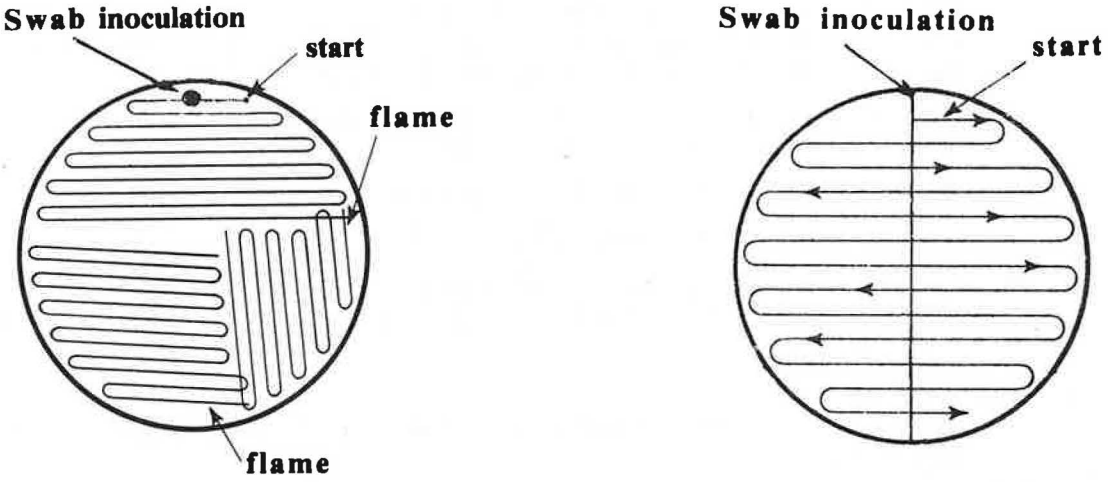
One (1) Nutrient agar deep
One (1) Petri plate
Three (3) Nutrient agar slants for isolated colonies

17

SWAB AND SPREAD PLATES

The swab plate and spread plate are alternative methods used to obtain isolated pure cultures. In many instances, specimens to be examined have been taken by swab and contain a high concentration of microorganisms. To directly swab the surface of the plate would result in confluent growth. The method of choice depends on the source of the specimen and usually involves the use of both the swab and the inoculating loop. Two swab methods, routinely used in clinical laboratories for isolation from throat and urine specimens, demonstrate these considerations.

Swab Culture Techniques



A. Throat culture method **B. Urine culture method**

The spread plate offers a rapid means of spreading a liquid inoculum over the surface of an agar plate. One loopful to 0.1 ml of the mixed culture is placed on the agar plate surface. A glass bent-rod is sterilized by placing the end in alcohol and igniting the tip. When cool, the bent-rod is used to spread the inoculum by rotating it around the surface of the plate.

Materials:

Broth working cultures of assigned organisms.
Two (2) Nutrient agar deeps
Two (2) Petri plates
One (1) sterile swab
One (1) bent-rod

NOTE: If there this a doubt about the purity of your culture, have your instructor examine them. Colonies you isolate from your cultures will be transferred to nutrient broth and slant tubes and used in Experiment 19: Cultural Characteristics.

18

POUR PLATE

The pour plate is an effective means of distributing a bacterial suspension evenly throughout an agar plate. While it may produce isolated colonies, depending on the concentration of bacterial cells, it is not typically used as a pure culture technique. It has many applications in research and industrial laboratories where procedures that determine the number of viable bacterial cells in various products, such as food or cosmetics, are required. A heavily inoculated pour plate provides an excellent surface on which to test bacterial reactions to antibiotics, disinfectants, chemicals, etc. Pour plates may be prepared using either of two methods.

1. A measured amount of the bacterial suspension is placed directly in a Petri plate. A melted nutrient medium, cooled to 45°C is poured into the plate, the cover replaced, and the plate gently rotated to achieve uniform distribution of the microorganisms; ***or***
2. The bacterial suspension is placed in a melted nutrient medium that has been cooled to 45°C and the tube is rolled rapidly between the palms of the hands to mix and then poured into a Petri plate.

Materials:

Broth working cultures of the assigned organism.
One (1) nutrient agar deep.
One (1) Petri plate
One (1) pipet (1 ml).
Small beaker of 70% alcohol per desk
Sterile paper disks (1.5 cm diameter)
Disinfectant (desk wash)

Method:

1. Melt the agar deep and place it in the 45°C water bath.
2. Select one of the methods outlined above.
3. Using 0.5 ml of the bacterial suspension, prepare one pour plate. Allow it to solidify completely.
4. Dip the forceps into the alcohol and ignite to sterilize.
5. Pick up a sterile paper disk with the forceps and touch the edge of the disk to the disinfectant, allowing it to absorb the disinfectant.
6. Lift the cover of the pour plate and place the saturated disk in the center of the agar surface. Close the cover.

19
CULTURAL CHARACTERISTICS

When microorganisms are cultured on a variety of media, they exhibit unique and identifiable *cultural characteristics*. In the classical method of identifying microorganisms, it is customary to determine these characteristics by examining the growth of the organism on nutrient agar plates and slants and in nutrient broth and nutrient gelatin. In this experiment, the cultural characteristics of the assigned bacterial species will be examined and described using the following illustrated descriptive terms:

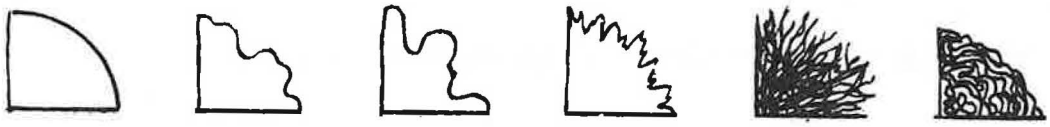
CULTURAL CHARACTERISTICS

Nutrient Agar Plates. Well isolated colonies are evaluated in the following manner:

1. Size: Pinpoint, small, moderate, or large.
2. Pigmentation: Color of colony.
3. Form: The shape of the colony.

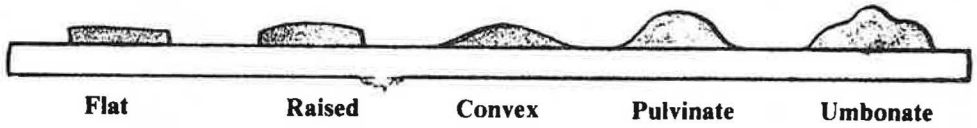
Punctiform **Circular** **Filamentous** **Irregular** **Rhizoid** **Granular**

4. Margin: The appearance of the outer edge of the colony.



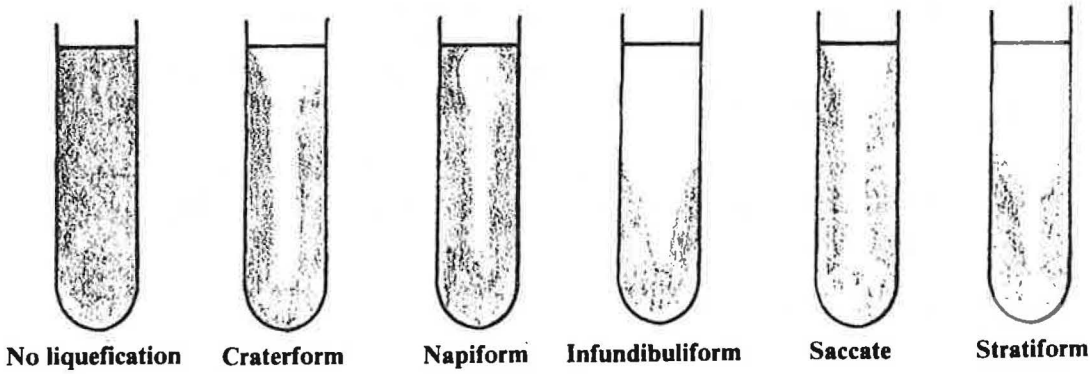
Entire **Undulate** **Lobate** **Erose** **Filamentous** **Curled**

5. Elevation: The form and degree to which colony growth is raised on the agar surface.



Flat **Raised** **Convex** **Pulvinate** **Umbonate**

Nutrient Gelatin. Liquified by bacterial enzymatic action. Liquefaction (hydrolysis) occurs in various patterns.



Bergey's Manual of Determinative Bacteriology, **8th ed.** contains the cultural characteristics of all described bacteria.

Materials:

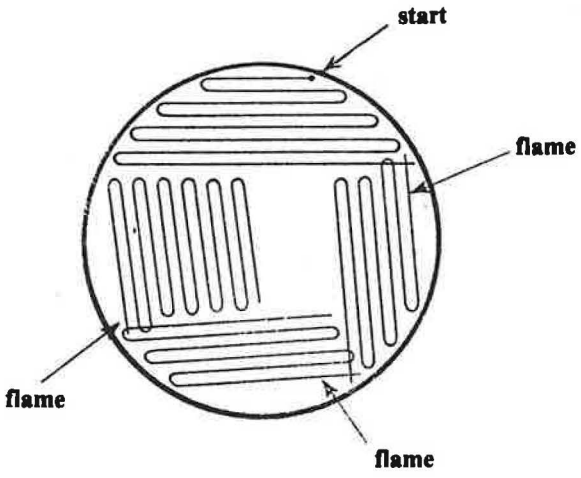
Broth and slant cultures of the assigned organisms.
One (1) nutrient broth
One (1) nutrient gelatin deep
One (1) nutrient agar deep
One (1) nutrient agar slant
One (1) Petri plate

Methods:

A. Transfer of a slant culture to a nutrient gelatin deep

1. Pick up the slant culture and the nutrient gelatin deep and hold in the left hand.
2. Flame the inoculating needle.
3. Remove the cotton plugs from the tubes and hold them between the fingers of the right hand. Sterilize the mouths of the tubes.
4. Hold the tubes almost parallel to the desk top to reduce the possibility of air-borne contaminants.
5. Touch the needle to the medium in the slant culture tube to be sure it is cooled and then touch the culture mass. Remove the needle without touching the sides of the tube.

6. Raise the cover of the Petri plate and follow the illustrated streak pattern below, reflaming the loop at each turn. This pattern is used when the concentration of microorganisms is high.It should produce well-isolated colonies.



7. Replace the agar plate cover and sterilize the inoculating loop.
8. Label the plate and incubate, inverted, at 21°C until the next laboratory.
9. Log the inoculation.

Observations:

1. Examine and describe the cultural characteristics of your organism using the descriptive terms given in the chart.
2. The nutrient gelatin culture must be chilled in the refrigerator for five (5) minutes before the determination of the pattern of liquefaction can be determined. Do not shake the tube before chilling. To examine the cooled tube for gelatin hydrolysis, tilt the tube and observe the flow pattern, if present. Classify the pattern using the terms given.
3. Record the cultural characteristics of your organism for future reference. These observations will provide the basis for the identification of unknown organisms.
4. Examine the cultural characteristics of other bacterial cultures. Note especially any unique qualities of these organisms.

20

SERIAL DILUTION-PLATE FOR VIABLE CELL COUNTS

Many methods have been developed to determine the number of bacterial cells in substances such as food, water, various commercial products, and the environment. These include direct microscopic counts, electronic counters, measurements of bacterial metabolic activity, estimation of dry weight, turbidimetric measurements of increases in cell mass, and the serial dilution plate method. The major disadvantage to some of these is that the total counts include both dead and living cells. Many studies, particularly those involving sanitary and, in some cases medical evaluations, require the determination of the numbers of viable cells. This is generally accomplished by using the serial dilution plate method. The procedure involves precise serial dilutions of a bacterial suspension to reduce the number of organisms to a point where they might be plated and effectively counted. Aliquots of the diluted suspensions are plated out on a suitable nutrient medium using the pour plate technique. The viable cells produce colonies on and in the medium and may be counted on the *Quebec colony counter.*

Plates suitable for counting must contain between 30 to 300 colonies to be statistically significant. The total viable cell count of the sample is obtained by multiplying the number of colonies per plate by the dilution factor, which is the reciprocal of the dilution.

Number of cells per ml = number of colonies X dilution factor

Example:

Colonies per plate = 75
Dilution factor = 1:1,000,000 ($1:1X10^6$)
75 X 1,000 = 75,000,000 cells per ml

Materials:

24-Hour broth culture of *Escherichia coli.*
Two (2) dilution bottles with 99 ml sterile physiological saline.
Two (2) test tubes with 9 ml of sterile physiological saline.
Four (4) nutrient agar deeps
Four (4) Petri plates
Four (4) pipets (1 ml)

4. Thoroughly mix the *Escherichia coli* culture by rolling the tube between the palms of your hands.

5. Aseptically transfer 1 ml of the sample to Bottle 1 and discard the pipet in disinfectant. The culture has now been diluted 100 times.

6. Mix Bottle 1 and, with a fresh pipet, transfer 1 ml to Bottle 2. Discard the pipet. The culture has not been diluted 10,000 times.

7. Mix Bottle 2 and transfer 1 ml to Pour Plate 1 using either of the two pour plate procedures you prefer. Also transfer 1 ml to Tube 3 and discard the pipet. The culture has now been diluted 100,000 times.

8. Mix Tube 3 and, with a fresh pipet, transfer 1 ml to Pour Plate 2, then transfer 1 ml to Tube 4. Discard the pipet. The culture has now been diluted 1,000,000 times.

9. Mix Tube 4 and, with a fresh pipet, transfer 1 ml to Pour Plate 3 and 0.1 ml to Pour Plate 4. By using only 0.1 ml for Pour Plate 4 you have increased the dilution to 10,000,000 times. You now have four (4) pour plates, each with the dilution indicated in the chart.

10. Once the agar has solidified, incubate the plates at 37°C for 24 hours and then at 21°C until the next laboratory session.
11. Examine all colonies on the plates using a Quebec colony counter. Plates with less than 30 colonies are designated as **too few to count (TFTC)** and plates with more than 300 colonies are designated as **too numerous to count (TNTC)**. Count only those plates containing 30 to 300 colonies. Count the subsurface as well as the surface colonies.
12. Calculate the number of viable cells per ml of the original sample. All team results will be compared and the average determined.

CULTIVATION OF MICROORGANISMS

The cultivation of microorganisms under laboratory conditions requires knowledge of their diverse nutritional and physical requirements and the specific requirements of the laboratory investigations. The next series of experiments will explore these three aspects of the cultivation of microorganisms.

Nutritional Requirements of Microorganisms

Though the sources may differ greatly, all organisms, including microorganisms, must have a source of **energy**, either radiant or chemical: **carbon** from carbohydrates and carbon dioxide; **nitrogen** from organic compounds, inorganic salts, ammonia, nitrates, and atmospheric nitrogen; **hydrogen** and **oxygen** from organic and inorganic compounds, water, and the atmosphere; **trace elements** from inorganic salts; **vitamins** through synthesis or compounds present in the medium; and **water**. The nutritional requirements among microorganisms vary from those of the autotrophs which can grow in wholly inorganic media to those of the obligate parasites that are so fastidious they cannot be grown in any artificial media.

Nutritional differences are essentially determined by the genetic make up of the organism. To obtain and utilize nutrients for synthesis and growth, an organism must be able to carry out chemical reactions by means of specific, genetically determined enzymes. Based on these enzymatic capabilities, nutrients are divided into two groups: **essential nutrients** which are those compounds the cell cannot synthesize, and **non-essential nutrients** which are useful but not indispensible. Culture media is formulated to supply or withhold the essential nutrients of microorganisms depending on the purpose of the laboratory investigation.

21
PREPARATION OF MEDIA

The nutrient formula of a medium is based on the organic and inorganic requirements of specific microorganisms or the specific function the medium is to serve. In this experiment, two types of media will be prepared: synthetic media designed to test the minimal growth requirements of certain bacteria and a general purpose medium, nutrient agar, formulated to satisfy the requirements of a wide variety of heterotrophic bacteria.

Materials:

Agar-agar
Ammonium phosphate
Glucose
Magnesium sulfate
Potassium chloride
Peptone (or other intermediate product of protein digestion)
Dehydrated nutirent agar
Graduated cylinder (1 liter)
Beaker (1 liter)
Stirring rod
Distilled water
Hot plate
Wire basket
Fifteen (15) test tubes and cotton plugs

Methods:

1. The following media will be prepared for use during next laboratory:

   a. **Agar-Agar**
      Agar-agar—15 g
      Distilled water—1000 ml

   b. **Agar + Minerals**
      Agar-agar—15 g
      Ammonium phosphate—1 g
      Potassium chloride—1 g
      Magnesium sulfate—1 g
      Distilled water—1000 ml

22

MINIMAL GROWTH REQUIREMENTS

Microorganisms have the same basic requirements for the synthesis of complex organic compounds and for the performance of life functions as all living organisms. However, there is great diversity in the compounds they can use. Some use carbohydrate (sugar and starches) as their source of energy. Many use protein and amino acids as their nitrogen source while others use ammonium phosphate, potassium nitrate, and other inorganic nitrogen compounds.

To determine the minimal growth requirements for nitrogen, for example, the bacterium is provided with media containing all its nutritional requirements but nitrogen. Various nitrogen compounds, or the like are added signle to determine if growth can occur. The presence of growth indicates the nutritional requirement or enzymatic capabilities of the bacterium.

Materials:

Slant cultures of:

Bacillus subtilis
Rhodospirillum rubrum
Pseudomonas fluorescens
Sarcina lutea

Four (4) Petri plates

One (1) deep of each medium prepared during the last laboratory:

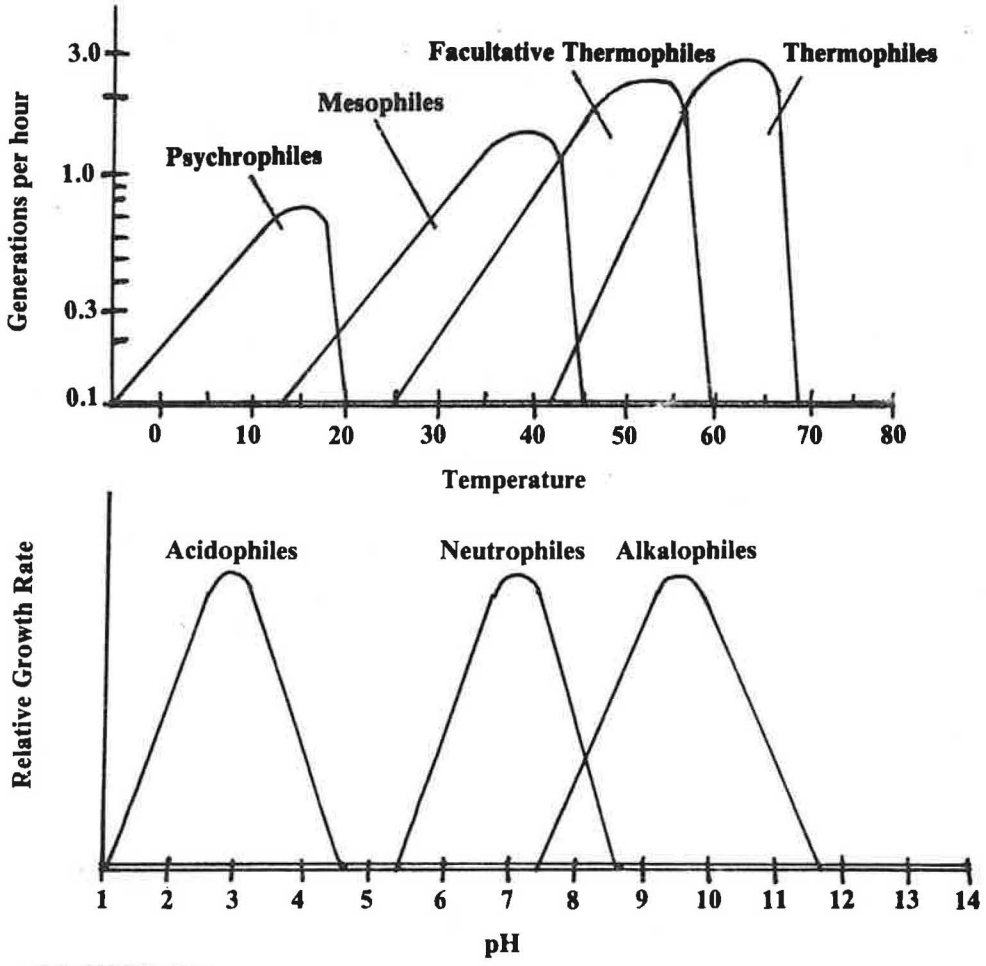
Agar-agar
Agar-minerals
Agar-minerals-carbohydrate energy
Agar-minerals-carbohydrate energy-organic nitrogen

Methods:

1. Work in teams of four (4) members.
2. Melt and pour one plate of each of the four minimal growth test media. Allow to solidify completely.
3. Divide the bottom of the plate into four sections and label each section with the name of one of the four test organisms.
4. Inoculate the corresponding sections of each plate with the appropriate organism.
5. Incubate at 21°C until next laboratory session.
6. Observe the amount and type of bacterial growth on each plate. Explain the results in relation to bacterial nutrition. Explain the nutritional requirements of an organism that grows poorly on all four media.

PHYSICAL REQUIREMENTS OF MICROORGANISMS

Microorganisms are profoundly influenced by physical environmental factors such as temperature, pH and oxygen availability. Though the majority of microbes require the same conditions as other forms of life, there are some that tolerate extremes in environmental conditions. Each species has a particular range of environmental conditions in which it can function and is classified according to its range. The following graphs illustrate the classification of bacteria by means of their range of tolerance to temperature and pH. The optimal, minimal, and maximal tolerances are indicated by the growth curve. Note that optimal growth occurs within a very limited range within the organisms total range.



Such information is important to the microbiologist for two reasons: (1) the environmental conditions must be adjusted to the maximum growth tolerances of each organism, and (2) specific organisms may be isolated by selectively manipulating the environmental factors. Bacteria are routinely isolated by such methods. For example: anaerobic pathogens which are often found in deep wounds may be isolated only in anaerobic conditions; systemic pathogens which generally can tolerate only the very limited range of pH found in the blood tissues must be cultivated in media within the blood pH range, and human pathogens typically require incubation at 37°C.

23
pH BUFFERS AND INDICATORS

One of the most important factors affecting enzymatic action is pH. Enzymes have optimum pH ranges in which biochemical reactions occur. Above or below this optimum pH, reactions are slowed down or stopped completely. Though the acidophiles and alkalophiles tolerate extremes in pH, most bacteria exhibit maximum growth between 6.5 and 7.5. As this narrow pH range about neutral is beneficial for the growth of most microorganisms, the pH of laboratory medium is frequently adjusted to this range.

As microorganisms grow within a medium, their metabolic wastes often cause pH changes that slow the rate of chemical reactions thereby affecting the growth rate and, ultimately, survival. Because of such changes, it is difficult to keep the pH of culture media constant during growth. **Buffers**, compounds that tend to prevent marked changes in pH, are added to retard these pH shifts.

The following experiment is designed to demonstrate the buffering effect of several solutions and the function of **pH indicators**, dyes which exhibit specific color changes in the presence of acids or bases. Phenol red is frequently used in microbiology laboratories. It is red in solutions with a pH of more than 6.9 and yellow in acidic solutions with a pH of less than 6.8.

Materials:

0.1 N HCl and 0.1 N NaOH solutions
0.2 M K_2HPO_4 and 0.2 M KH_2PO_4 buffer solutions
0.02 percent phenol red indicator solution
One (1) nutrient broth
Four (4) test tubes
Pipets: 10 ml (4); 1 ml (1)
Distilled water

Methods:

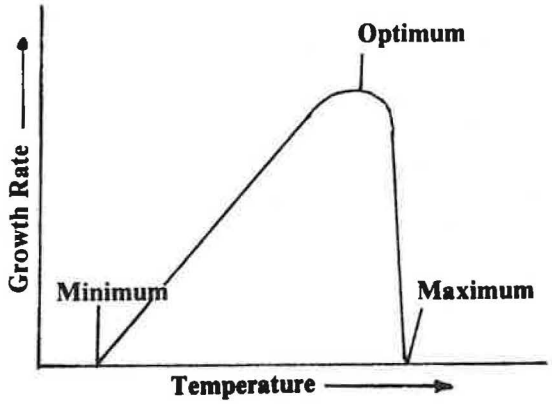
1. Work in teams of two (2) members with one set of reagents per desk. Aseptic techniques are not required for this study.
2. Number four (4) test tubes 1 through 4 and set in a rack.
3. Pipet 5 ml distilled water into Tube 1. It is the control.
4. Pipet 1 ml of K_2HPO_4 buffer solution into Tube 2.
5. Pipet 1 ml of KH_2PO_4 buffer solution into Tube 3.
6. Pipet 5 ml of nutrient broth into Tube 4.

24
TEMPERATURE RELATIONSHIPS

The growth and survival of microorganisms are greatly influenced by the temperature of the environment. Temperature affects living organisms in one of three ways: (1) low temperatures may produce a bacteriostatic effect where no growth occurs or cause death through inactivation of enzymes or disruption of the cell structure, (2) with an increase in temperature, chemical and enzymatic reactions occur at an increased rate and synthesis and growth are faster or (3) cellular components, proteins and nucleic acids sensitive to high temperature, can become inactivated or destroyed causing cellular death. Therefore, as the temperature increases, metabolism and growth increase up to a lethal point where cell function ceases.

Within the temperature range of the species, there is a minimum temperature below which no growth occurs, an optimum temperature with rapid growth, and a maximum temperature above which life functions cease. These are the **cardinal temperature points** and are characteristic for each organism though they are modified by such environmental factors as pH and nutrition.

Cardinal Temperature Points



Materials:

Broth working culture of your assigned organism
Four (4) nutrient agar slants

Methods:

1. Label each of the slants with the name of your culture and the temperature of incubation (4, 21, 37, 60 degrees C).

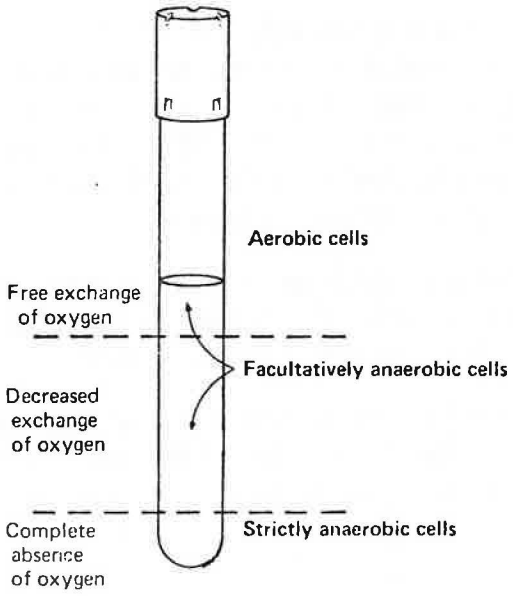
2. Inoculate the tubes and incubate at the appropriate temperatures until next laboratory.

3. Observe the amount of growth and classify your organism as to its range of temperature tolerances. If an organism grew at 20°C, how would you determine experimentally whether the organism was a psychrophile or a mesophile?

25

ATMOSPHERIC OXYGEN REQUIREMENTS

Bacteria demonstrate considerable variation in their responses to gaseous oxygen. They are typically classified into three major groups: aerobes, facultative anaerobes, and anaerobes. While most aerobes and facultative anaerobes are easily cultivated in the laboratory, obligate anaeorbes require special procedures for recovery, cultivation, and identification. Certain of these procedures have only recently been developed and have greatly increased our knowledge and appreciation of the importance of anaeorbes in infectious diseases. Anaerobes make up more than 50 percent of the total population in many areas of the body and are most likely involved in infections in the blood, abscesses, deep wounds, spinal and synovial fluids, and contaminated food. The few aerobes and facultative anaerobes that are indigenous or parasitic to humans, such as the coliforms *Escherichia* and *Aerobacter*, require high concentrations of carbon dioxide for growth. Such carbon dioxide requiring bacteria are called **capneic**.



Agar Deep Culture

The following four (4) methods will be used to evaluate the oxygen and carbon dioxide requirements of selected bacteria:

GasPax Method consists of a jar with alid that is clamped down, a catalyst located in the lid, and a replaceable envelope congaining hydrogen-generating chemicals that are activated when water is added to the envelope. When the water is added to the envelope, any oxygen in the jar reacts with the hydrogen gas and in the presence of the catalyst produces water. The system also contains a methylene blue indicator strip which is colorless under anaerobic conditions.

Methods:

1. Work in teams of four (4) members.
2. Each team of four will be assigned four bacteria. Each member will culture only one of the bacteria. Have a Working Culture of your assigned Stock Culture ready for inoculations.
3. Melt ten (10) nutrient agar deeps and cool to 45°C in the water bath.
4. Label all plates and tubes.

   A. **Thioglycollate Broth**
      1. Inoculate a thioglycollate broth with one loopful of your test culture.
      2. Incubate at 21°C until the next laboratory.

   B. **GasPax and Candle Jar Methods**
      1. Pour and cool two (2) Petri plates per team.
      2. Using a wax pencil, mark the bottom of the two plates into four (4) sections and label each section with the name of one of the four bacterial cultures.
      3. Inoculate your test culture into the appropriate section on each plate and have your team members do the same until all sections are inoculated.
      4. Place one inoculated plate in the GasPax and the other in a Candle jar. These will be incubated at 21°C and returned to you during the next laboratory.

   C. **Shake-Culture Method**
      1. Inoculate a melted and cooled agar deep with one loopful of your culture.
      2. Shake thoroughly. Do not permit the cotton plug to become moist. Set aside to solidify.
      3. Using a Working Culture of your Stock Culture, inoculate one loopful into a melted and cooled agar deep, shake thoroughly, and set aside to solidify.
      4. Incubate both tubes at 21°C until next laboratory session.

TYPES OF MEDIA BASED ON LABORATORY USE

Knowing the nutritional and physical requirements ofmicroorganisms, it is relatively simple to prepare specific media to isolate and examine the particular characteristics of bacterial species. Many such media have been formulated and are readily available to fill the needs of most laboratories. Culture media can be classified according to their chemical composition and use.

Chemical Composition:

Synthetic Media. In synthetic media, the exact composition is known or chemically defined. It may contain only inorganic salts with a carbon source or many organic compounds. It is used, for example, in precise research where variables must be controlled, for the culture of autotrophs or, as in Experiment 22, to determine the minimal growth requirements of a bacterium.

Non-Synthetic Media. In non-synthetic media the exact chemical composition is not known. It contains tissue extracts or beef broths. Nutrient broth is a non-synthetic medium.

Purpose:

Enrichment Media. Enrichment media are basal media supplemented with additional nutrients to support the growth of specific fastidious bacteria in a mixed culture. These are commonly used in clinical laboratories to enhance the growth of fastitious pathogens.

Selective Media. Selective media serve much the same function as enrichment media except that they contain agents that inhibit the growth of unwanted organisms in mixed cultures. For example, an inhibitory agent, such as a dye or penicillin may be added which would inhibit the growth of Gram-positive but not Gram-negative organisms.

Differential Media. Differential media are designed to differentiate closely related bacteria on the basis of cultural characteristics and biochemical reactions within the media. Methylene-Blue Agar is both a selective and a differential medium. It supports the growth of Gram-negative bacteria and differentiates between *Eschericia coli* and *Enterobacter aerogenes. E. coli* colonies produce a metallic sheen while *Entero. aerogenes* colonies are pink and viscous.

General Purpose Media. These media are designed to support the growth of most microorganisms commonly used in microbiology laboratories. Examples are nutrient and trypticase-soy broths or agar.

26
SELECTIVE and DIFFERENTIAL MEDIA

The isolation of a particular organism may involve the use of several media before a pure culture is finally obtained. For example, to isolate a Gram-negative bacillus, a medium that inhibits Gram-positive but not Gram-negative organisms might be used first. Once the Gram-negative colonies are obtained they might be transferred to another selective media that inhibits certain of the Gram-negative forms or a differential medium that permits distinguishing organisms on the basis of macroscopic colony characteristics.

When differentiating bacteria as to their species classification, biochemical reactions become important. Differential media distinguish among bacteria on the basis of chemical reactions as well. For example, media containing a pH indicator will change color when acid or alkaline metabolic wastes are produced due to the enzymatic activity of certain bacterial species.

Materials:

Broth cultures of:
- *Alkaligenes viscolactis*
- *Sarcina lutea*
- *Escherichia coli*
- *Bacilus subtilis*

One (1) nutrient agar deep
One (1) sodium chloride agar deep
One (1) phenol red dextrose agar deep
One (1) Crystal violet agar deep (1:100,000 dilution)
Four (4) Petri plates

Methods:

1. Work in teams of four (4) members.
2. Melt and pour one plate each of the four types of media.
3. Divide the bottom into sections and label each section with the name of one of the four bacterial test cultures.
4. Inoculate the corresponding section of each plate with the appropriate organism.
5. Incubate at 21°C until next laboratory.
6. Observe the plates and explain the reactions. Which of the four media was selective? Differential? How would you further isolate these organisms if these media did not produce a pure culture of the desired organism?

27
PRIMARY ISOLATION MEDIA

Most clinical specimens contain a mixed flora of microorganisms. Depending on the source and the expected infectious agents, numerous media, called **primary isolation media**, are inoculated. These serve several purposes: (1) to determine the predominating species; (2) to differentiate species, and (3) to selectively encourage the growth of the suspected pathogens.

The physical requirements of organisms (temperature, pH, and oxygen) function as selective agents also and are consistently used to isolate specific microorganisms.

Materials:

Simulated fecal suspension (SFS) containing:
- *Escherichia coli*
- *Pseudomonas aeruginosa*
- *Staphylococcus epidermidis*

Demonstration streak plates of:
- *Escherichia coli*
- *Staphylococcus aureus*

One (1) prepared blood agar plate
One (1) Eosin methylene blue agar deep (E.M.B.)
One (1) Mannitol salt agar deep (M.S.A.)
One (1) MacConkey agar deep
Two (2) Trypticase soy agar deeps
Five (5) Petri plates
Three (3) sterile water blanks
Three (3) sterile swabs
One (1) urine specimen cup
One (1) sterile towelette
Difco Manual or *BBL Manual of Products and Laboratory Procedures*

Methods:

A. Primary Isolation of Microorganisms

1. Divide four (4) Petri plates and the blood agar plates into four (4) sections each and label them: nose, throat, urine, and S.F.S.

2. Melt the agar deeps and pour the E.M.B., M.S.A., MacConkey, and one of the trypticase-soy agar deeps into the previously section Petri plates. The remaining melted trypticase-soy deeps should be kept in the 45° C water bath ready for preparation of a urine specimen pour plate, in Step 6.

3. Swab your throat and inoculate each of the five sectioned plates in the proper area.

Methods:

1. Work in teams of four (4) members.
2. Make a table of the above media and indicate the classification, the physical factors, selective or differential agents, and the organisms isolated. Use the table below as a format.
3. How would you isolate a Gram-negative enteric bacilli? An anaerobic *Neisseria*? A hemolytic *Streptococcus*? *Staphylococcus aureus* from a pus-forming lesion? When would Thayer-Martin agar be used rather than chocolate agar?

Primary Isolation Media

Media	Type	Physical Factors	Selective and Differential Agent(s)	Type of Organisms Isolated
Phenylethyl alcohol agar (PEA)	Selective	35°C pH 7.2	Phenylethyl alcohol (inhibits Gram-negatives)	Gram-positive cocci
Thayer-Martin agar (TM)	Selective	35°C pH 7.2	Hemoglobin, growth factors, and antibiotics	Pathogenic *Neisseria* species

BIOCHEMICAL CHARACTERISTICS OF MICROORGANISMS

All of the activities of the microbial cell are mediated by species specific enzymes. In many cases, **exoenzymes** are secreted into the environment where they modify macromolecules releasing smaller molecules that may enter the cell. There, **endoenzymes** utilize them as energy sources or building blocks for synthesis of cell constituents and in the process, metabolic wastes are produced and released into the environment. Most tests done in microbiology laboratories measure these activities, determining the cell's ability to produce specific enzymes, metabolites, and end products. The identification and classification of bacteria is to a considerable extent based on such changes produced by pure cultures growing in or on various differential media.

The following experiments will use routine methods to determine the biochemical characteristics of the assigned bacterial cultures. These bacteria are representative types and will serve as the basis for the identification of unknown organisms.

The biochemical characteristics of microorganisms and the media used are outlined below. Review the purpose of these media in either *Difco Manual* or *BBL Manual of Products and Laboratory Procedures* and the characteristic reactions of your bacterium in *Bergey's Manual of Determinative Bacteriology.*

BIOCHEMICAL ACTIVITY	MEDIA	REAGENTS/INDICATORS
I Exoenzymatic Activities		
Starch hydrolysis	Starch agar plate	Iodine
Lipid hydrolysis	Tributyrin agar plate	Neutral red
Casein hydrolysis	Casein agar plate	None
Gelatin hydrolysis	Nutrient gelatin deep	None
	Gelatin agar plate	Picric acid
II Endoenzymatic Activities		
Carbohydrate fermentations	Fermentation tubes	Phenol red
	Fermentation disk plate	Phenol red
Litmus milk reactions	Litmus milk tubes	Litmus
Nitrate reduction	Tripticase-nitrate broth	Sulfanilic acid, alpha-naphthylamine, and zinc
Urease production	Urea broth	Phenol red
	Nutrient broth	Urease test tablets
Oxidase production	Trypticase-soy plate	Oxidase test paper
Catalase production	Trypticase-soy plate	Hydrogen peroxide
Battery Tests		
Hydrogen sulfide production	S.I.M. deep	Ferrous sulfate
	T.S.I. deep slants	Ferrous sulfate
Indole production	S.I.M. deep	Kovac's reagent
Motility	S.I.M. deep	None
Glucose fermentation	MR-VP	Methyl red
Acetylmethylcarbinol production	MR-VP	Barritt's reagent
Citrate utilization	Simmon's citrate slant	Brom thymol blue
Carbohydrate fermentations and H_2S production	T.S.I. deep slants	None

NOTE:

You will be using a variety of media, some of which look very similar. Be sure to label the tubes as you pick them up at the media cart.

28

HYDROLYSIS OF POLYSACCHARIDES, PROTEINS, AND LIPIDS

The macromolecules of starch, lipids, and proteins are insoluble and cannot be taken into the cell directly. Bacterial exoenzymes must be secreted to hydrolyze these molecules into smaller units: starch is broken down to monosaccharides and disaccharides; lipids to fatty acids and glycerol, and proteins to amino acids. These molecules can then pass through the cell membrane and be utilized. The hydrolysis of these substrates may be demonstrated by changes in the media.

Materials:

Slant cultures of:
- *Bacillus subtilis*
- *Escherichia coli*
- *Proteus vulgaris*
- *Pseudomonas fluorescens*
- Working Cultures of assigned organisms

Four (4) starch agar deeps
Four (4) Tributyrin nutrient agar deeps with neutral red indicator
Four (4) nutrient gelatin agar deeps
Eight (8) Petri plates
Dilute iodine solution

Methods:

1. Work in teams of four (4) members. Each member will use one of the prepared cultures and his/her working culture.
2. Each member will divide two plates into two (2) sections and label fully including the name of the medium. One plate will contain starch agar and the other tributyrin agar.
3. Melt and pour the media into the proper plate, allowing them to solidify completely.
4. Inoculate each section of each of the plates making one heavy streak.
5. Stab inoculate a nutrient gelatin deep with one of the test organisms. Do not shake or mix the contents of the tube.
6. Incubate at 21°C until next laboratory.

29

CARBOHYDRATE FERMENTATIONS

Most aerobic and anaerobic microorganisms use carbohydrates as their primary source of energy. Monosaccharides and disaccharides are oxidized by ordered sequences of enzymatic reactions or biooxidative pathways. By means of **cellular respiration,** with or without oxygen, or **fermentation,** the energy within the chemical bonds of the carbohydrate is released (for use by the cell) and various end-products are the result. Bacteria differ greatly in the end-products they produce from a given sugar. Some produce both acid and gas, while others produce only acid. Others produce neither.

Two methods will be used to determine the fermentative activity of bacteria: fermentation tubes and fermentation disks. Fermentations usually occur within 24 to 48 hours after inoculation. Extended incubation permits other enzymatic reactions to mask those of fermentation producing false negatives. The production of acid will cause the indicator, phenol red, to turn yellow. The gases that may be produced within the fermentation tubes will be trapped within the inverted Durham tube.

Materials:

Slant cultures of:
- *Bacillus subtilis*
- *Escherchia coli*
- *Staphylococcus aureus*
- *Alcaligenes viscolactis*
- Working Cultures of assigned organisms

Eight (8) fermentation tubes of:
- lactose
- glucose (dextrose)
- sucrose

Four (4) nutrient agar deeps containing 0.025 gm. phenol red per liter
Sterile 0.05 percent aqueous concentrations of selected sugars
Four (4) Petri plates
Forceps and alcohol

Methods:

A. Fermentation Sugar Disc Method

1. Work in teams of four (4) members. Each member will use one of the prepared cultures and his/her working culture.

2. Label the fermentation tubes as you pick them up from the media cart.

3. Using 1 ml of your working culture, prepare a phenol red nutrient agar pour plate. Allow it to solidify completely.

30
LITMUS MILK REACTIONS

Litmus milk is an excellent media for growth and differentiation as it will support the growth of a variety of microorganisms. It contains the milk sugar, lactose; the milk proteins, casein, lactoalbumin, and lactoglobulin; vitamins, and minerals. Litmus is added as an indicator as well as a reducible dye. As an indicator, it turns pink in the acid condition and more deeply purple in the alkaline condition. As a reducible dye, it loses its color and becomes creamy white. Because of the nature of the medium, a variety of reactions may occur:

1. **Lactose fermentation** occurs when an organism uses lactose as a carbon source of energy. The metabolic pathway is through glycolysis to lactic acid. Lactic acid production is detected when litmus turns pink (at approximately pH 4) and an acid curd forms in the milk. Acid curds are firm and remain in place when the tube is tilted.

2. **Reduction of litmus** is due to the oxidative activities of the bacterium and is indicated by the loss of litmus color. This is most apparent when a pink acid curd begins to turn white.

3. **Gas formation,** as an end-product of lactose fermentation, may include carbon dioxide and hydrogen. Evidence of gas may be seen as breaks or fissures within the acid curd.

4. **Rennin curd production** will occur when certain bacteria produce enzymes that form an insoluble calcium-casein complex. Unlike the acid curd, rennin forms a soft, semisolid curd that flows slowly when the tube is tilted.

5. **Proteolysis** occurs when some microorganisms, unable to obtain energy by way of lactose fermentation, hydrolyze the milk proteins to their basic units, aminio acids. As the proteins are digested, the medium begins to clear starting at the top of the tube and proceeding downward. In addition, the medium becomes brownish and translucent.

6. **Alkaline Reaction** is also due to the utilization of casein. Ammonia, which is highly soluble in water, and other basic substances accumulate causing the litmus to change from light purple to deep purple.

7. **Ropiness** is the result of the growth of such organisms as *Alkaligenes viscolastis.* The cells become encapsulated due to the highly nutritive environment and form a slimy growth in the medium. This "ropiness" can be detected by testing the medium with an inoculating loop and observing long "ropes," or masses of cells.

31
NITRATE REDUCTION

Microorganisms are versatile in their ability to utilize different sources of nitrogen. Some are able to convert atmospheric nitrogen into forms that are precursors to organic molecules, while others degrade organic wastes releasing essential materials into the environment. Intermediate to these is a wide range of microbial activities that not only make nitrogen available to microorganisms but evolve forms of nitrogen useable by other organisms. Because of these diverse enzymatic capabilities, microorganisms play an important role in the nitrogen cycle by maintaining the flow of nitrogen throughout the biosphere and providing all forms of heterotrophic life, ourselves included, with essential sources of nitrogen.

Bacteria differ in their ability to use a specific form of nitrogen, for example nitrates. Some can reduce nitrates to nitrities. Others can reduce nitrates to the ammonia and gaseous nitrogen. Still others do not reduce nitrates at all. These various reactions provide additional means for the differentiation and identification of bacterial types. The reduction of nitrate and the subsequent reduction of nitrite can be detected by growing a bacterium in nutrient broth to which potassium nitrate has been added. One of the following results may occur depending on the enzymatic capabilities of the bacterium:

- The nitrate may remain unaltered.
- Nitrate may be reduced to nitrite.
- Nitrate may be reduced to nitrite and then further reduced to ammonia or free nitrogen.

Materials:

Broth cultures of:
- *Excherichia coli*
- *Pseudomonas aeruginosa*
- *Proteus vulgaris*
- *Bacillus subtilis*
- Working Cultures of assigned organism

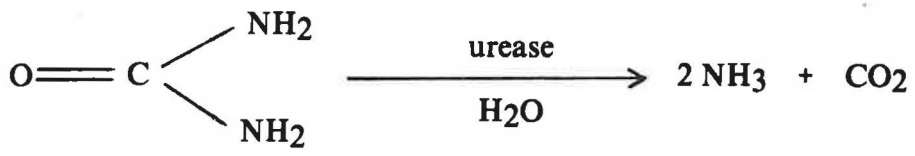
Nine (9) Trypticase-nitrate broths

Nitrite reagents:
- Sulfanilic acid solution
- Dimethyl-alpha-naphthylamine solution

Zinc powder

32
UREASE PRODUCTION

The interdependence of organisms becomes most evident when examining the physiology of microbes. Many organisms are able to synthesize amino acids from the by-products of carbohydrate and lipid metabolism if they are provided with ammonia as a source of nitrogen. Many other organisms are able to convert various forms of inorganic oxidized nitrogen to ammonia. Some bacteria are able to convert urea, an organic waste product of animal metabolism, into ammonia and carbon dioxide.



The ability of microorganisms to produce urease and effect urea hydrolysis is used to differentiate the genus *Proteus* from other enteric Gram-negative organisms, especially enteric pathogens such as *Salmonella* and *Shigella* which it closely mimics. The presence of urease production may be detected using **urea broth**, or agar containing nutrients, urea, and phenol red. As urease positive organisms hydrolyze urea releasing ammonia, the pH of the medium becomes alkaline and the indicator turns deep red.

Materials:

Broth cultures of:
- *Escherichia coli*
- *Proteus vulgaris*
- Working Cultures of the assigned organisms

Six (6) urea broth tubes
One (1) nutrient broth

Methods:

Work in teams of four (4) members in both studies.

1. Label urea broth tubes. Inoculate two with the test cultures and the other four with the Working Cultures of team members.
2. Incubate 24 to 48 hours at 37°C.
3. Examine the urea broth culture tubes and note any changes in the indicator color. An uninoculated urea broth may be used for comparison. Urease production is indicated by a change in color to deep red. No change in color occurs in tubes inoculated with urease-negative organisms.

33

OXIDASE AND CATALASE PRODUCTION

Oxidase and catalase are important in the differentiation and identification of certain microorganisms. Both are associated with the electron transport system in aerobic organisms. *Cytochrome oxidase* functions in the transport of the hydrogen atom to oxygen resulting in the formation of water by some organisms and hydrogen peroxide by others. All aerobic, as well as some facultative anaerobes and micro-aerophiles, produce cytochrome oxidase. Those organisms tht produce hydrogen peroxide, a substance toxic to cells, also produce *catalase*, an enzyme that breaks down hydrogen peroxide to oxygen and water.

$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2\ H_2O + O_2$$

The oxidase test is useful in the differentiation of members of the genera *Neisseria* and *Pseudomonas* which are oxidase-positive and the family Enterobacteriaceae which are oxidase-negative. The catalase test identifies the catalase-negative genera which includes *Streptococcus* and *Clostridium*.

Materials:

Broth cultures of:

- *Escherichia coli* ┐
- *Pseudomonas fluorescens* ┘ **Group 1**
- *Staphylococcus aureus* ┐
- *Streptococcus faecalis* ┘ **Group 2**
- Working Cultures of the assigned organisms

One (1) trypticase-soy agar deep
One (1) Petri plate
Three (3) oxidase test disks
Three (3) capillary tubes
Small beaker of hydrogen peroxide

Methods:

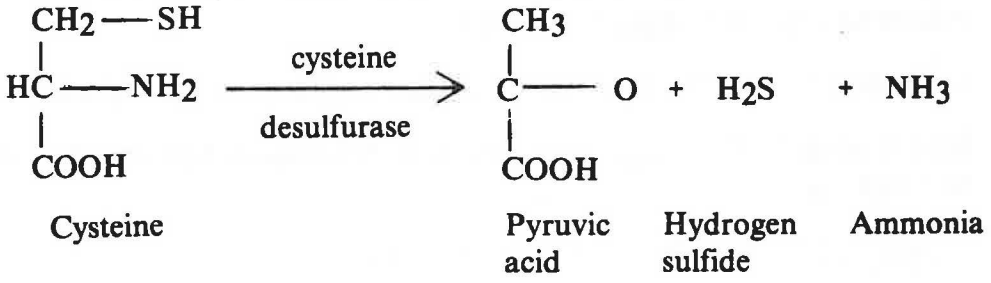
1. Work in teams of two (2) members. One member should use Group 1 organisms; the other Group 2 organisms.

2. Divide the Petri plate into three (3) sections and label with the names of the test organisms and your assigned organism.

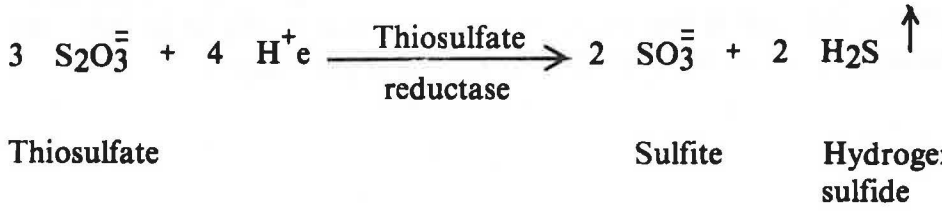
34

SIM TESTS

Bacteria are able to use sulfur-containing amino acids and inorganic compounds in the fermentative production of **hydrogen sulfide**. Some bacteria produce the enzyme *cysteine disulfurase*, which removes sulfur from the amino acid, cysteine, during the deamination of cyteine to pyruvic acid, and releases hydrogen sulfide.



Hydrogen sulfide is also produced by bacterial action in the reduction of inorganic sulfur compounds such as thiosulfate.



If metallic ions, such as iron, lead, or bismuth, are available, the hydrogen sulfide formed during growth combines with the metallic ions to form a metal sulfide that blackens the medium. Examples of this reaction are seen in cans with spoiled food and in sediments in some ponds. The characteristic odor is that of rotting eggs.

The bacterial production of the enzyme *tryptophanase* converts the amino acid, tryptophan, into *indole*, pyruvic acid, and ammonia. While indole is a putrefactive waste product, pyruvic acid and ammonia may be used within the cell for energy and protein synthesis. SIM medium is a convenient medium for testing hydrogen sulfide and indole production as well as motility determination. It contains peptones and thiosulphates as sources of the enzyme substrates, ferrous ammonium sulfate as a source of iron ions which function as a hydrogen sulfide indicator, and sufficient agar to promote anaerobic respiration and determine bacterial motility.

35

MR-VP TESTS

Glucose fermentation yields three types of end-products: gases, acids, and neutral substances. Though all enteric bacteria ferment glucose as their primary source of energy, the kind and amount of end-products vary according to the enzymatic pathways present in the bacteria. The MR-VP tests are designed to differentiate bacteria on the basis of these fermentative end-products. Bacteria that produce large amounts of **acids** from glucose lower the pH of the medium to below pH 4.5 where, because of the high acid concentration, further growth is limited. Other bacteria initially produce acids but the lowered pH triggers the induction of enzymes that convert the acids into neutral end-products such as *2,3 butanediol* and *acetylmethylcarbinol* causing the pH of the medium to increase to about 6. These differences in enzymatic reactions are the basis for the methyl red (MR) portion of the test.

Methyl red is an indicator with a pH range of 4 to 6 and is red at pH 4 or below and yellow at pH 6 or above. Bacteria that produce acids decreasing the pH to 4 and below turn the media red and are *methyl red-positive*, while those that have a final pH of 6 and above due to the subsequent conversion of the acids to neutral end-products turn the medium yellow and are *methyl red-negative.*

The Voges-Proskauer (VP) test works in conjunction with the methyl red test in that it measures the presence of the neutral end-products *2,3 butanediol* and *acetylmethylcarbinol* (acetoin) from glucose fermentation. Two reagents are used in the VP test to produce a color change: Barritt's reagent (alpha-naphthol) and 40% KOH. Alpha-naphthol reacts with acetoin or 2,3 butanediol in the presence of air and KOH to produce diacetyl. The diacetyl in turn reacts with a guanidine residue of arginine in the broth to produce a rose color.

Materials:

Slant cultures of:
- *Escherichia coli*
- *Enterobacter aerogenes*
- Working Culture of the assigned organism

Six (6) MR-VP broth tubes
Barritt's reagent
40% KOH

Methods:

1. Work in teams of four (4) members.
2. Label the tubes and inoculate with the appropriate organism.
3. Incubate at 37°C for 48 hours.

36
CITRATE UTILIZATION

In the absence of fermentable sugars, certain bacteria are able to ferment citrate as their sole source of carbon energy. These bacteria produce the enzyme **citrate permease** which transports citrate into the cell. There, by a series of enzymatic reactions, it is converted to pyruvic acid and carbon dioxide. Pyruvic acid may be reduced throgh fermentation yielding energy and a variety of end-products.

The production of citrate permease is an identifiable characteristic and may be determined by inoculating the bacterium into Simmon's Citrate agar, an organic synthetic medium, in which sodium citrate is the only source of carbon and energy. In this medium, the pH indicator, bromthymol blue, is a significant choice. The pH range of bromthymol blue is from pH 6.0 to 7.6, and is blue at the more alkaline end of the range and yellow at the more acidic end. it displays an intermediate green color at pH 6.9. As citrate-positive organisms grow on the Simmon's citrate, the carbon dioxide that is generated in the reaction combines with sodium and water to form sodium carbonate, an alkaline product, that turns the medium a rich blue.

Materials:

Broth cultures of:
- *Escherichia coli*
- *Enterobacter aerogenes*
- Working Culture of the assigned organism

Six (6) Simmon's citrate agar slants

Methods:

1. Work in teams of four (4) members.
2. Label the slants and inoculate each organism into the appropriate tube by using the stab-and-streak method. Stab to within 1 cm of the bottom of the tube and streak across the surface of the slant.
3. Incubate at 37°C for 24 to 48 hours.
4. Examine the slant cultures for the presence or absence of growth and the color of the medium. A change from green to blue is a positive test for the utilization of citrate as a carbon source.

37
TRIPLE SUGAR IRON AGAR TESTS

Triple sugar iron agar is a multipurpose medium which combines tests for the fermentations of glucose, lactose, sucrose, and the production of hydrogen sulfide. It is designed to distinguish the Enterobacteriacea from other Gram-negative bacilli and to efficiently differentiate the groups and genera of the Enterobacteriacea, which are are all Gram-negative bacilli that ferment glucose to acid.

Triple iron sugar agar is a slant medium prepared with a deep butt. Microorganisms are inoculated into and on the medium by the stab-streak method. There they may metabolize the sugars to acids by the glycolytic process. Phenol red is used as the indicator and turns yellow with acid production. The microorganisms may also metabolize sulfur-containing amino acids or thiosulfate in the medium producing hydrogen sulfide. Hydrogen sulfide combines with the iron ions in the medium to produce visible ferrous sulfide. Most microorganisms can grow on this medium but differ in their enzymatic capabilities to metabolize the sugars and amino acids. These differences result in characteristic changes in the medium:

1. **Alkaline slant and acid butt** indicate that glucose is the only one of the three sugars metabolized. The reasoning for this is:
   - The bacteria preferentially ferment glucose first.
   - The concentration of glucose in the medium is minimal, one-tenth the concentration of the other two sugars.
   - Air is available in the slant and permits the oxidation of the acids to neutral products.
   - The anaerobic conditions within the butt of the tube prevent the oxidation of the acids.
2. **Acid slant and acid butt** indicate that lactose and/or sucrose have been metabolized because:
   - Organisms that metabolize a sugar other than glucose will produce considerably more acid due to the high concentration of these sugars.
   - Oxidation in the slant area does not occur rapidly enough to produce enough neutral end-products to change the pH.
3. **Alkaline slant and alkaline butt** indicate that no carbohydrate fermentation has occurred because:
   - Some microorganisms cannot metabolize any of the sugars in the medium. Their carbon and energy sources are the peptones provided in the medium.
   - Peptones metabolized under aerobic and/or anaerobic conditions produce ammonia and result in an alkaline pH.

Materials:

Slant cultures of:

Pseudomonas aeruginosa
Escherichia coli
Proteus vulgaris
Alcaligenes faecalis
Working Cultures of the assigned organisms

Eight (8) triple sugar iron agar slants

Methods:

1. Work in teams of four (4) members.

2. Label and inoculate each tube with the appropriate organism using the stab-streak method.

3. Incubate at 37° C for 18 to 24 hours. In order to insure proper interpretations, the tubes must be read within 18 to 24 hours.

4. Read the tube and record your results, giving the color of the slant first and then the butt. Use A for acid (yellow) and K for alkaline (red); *e.g.*, K/A for alkaline slant and acid butt. Compre your results with the illustrations given.

38

ISOLATION AND IDENTIFICATION OF BACTERIA IN MIXED CULTURES

One of the major responsibilites of the microbiologist is the isolation and identification of unknown bacteria. Clinical specimens and samples of food, water, and various products are diligently examined to determine the causative agents of disease and the presence of contaminants. Countless materials are examined in the search for new microbial sources of useful products such as antibiotics, enzymes, vitamins, and solvents. You have now developed sufficient knowledge of isolation and staining techniques and the biochemical activities and characteristics of bacteria to be able to work independently in an attempt to isolate and identify unknown species in mixed cultures.

You will be given a mixed culture containing several bacteria selected from those previously studied as well as a few unfamiliar species. You will be required to isolate and identify each of the bacteria *using only that media necessary for identification.* In order to help you organize your laboratory tests, a **flow chart** will be prepared using the data collected from previous tests of assigned bacteria. The chart will be of assistance in planning the media you will use for the rapid and efficient identification of your unknowns. Keep up-to-date, accurate records in your log book. Leave a series of pages for each unknown so all data regarding each unknown may be easily located.

Materials:

Mixed culture
Two (2) nutrient agar deeps
Two (2) Petri plates
Media AS REQUESTED

Methods:

1. Prepare two (2) nutrient agar plates. Dry the plates in the incubator to remove excess moisture.

2. Immediately upon receipt of the suspension of your unknown mixture, streak each plate to obtain well-isolated colonies. Incubate one plate at room temperature; the other label with your name and unknown mixture number and place in in the **inverted** position in the tray provided. This plate will be incubated at 37°C for 24 hours, graded for your streaking technique, and then returned to you.

3. After you have streaked your plates, prepare a Gram stain of the unknown mixture. Observe and record the Gram reactions, the cellular forms, and the cell arrangement of the bacteria present.

CONTROL OF MICROORGANISMS

There are few places where microorganisms cannot be found and many places where it is essential that they not be found. While many bacteria are beneficial and, in fact necessary, others are pervasive, and can be destructive and lethal. The effective control of such bacteria is necessary to prevent or treat diseases, inhibit the spoilage of food and industrial products, and exclude their contaminating effects in precise research and development. Most methods of control involve physical and chemical agents that adversely affect bacterial structures and functions, thereby inhibiting their growth or killing them. These measures include the use of heat, radiation, antiseptics, disinfectants, antibiotics.

Though the mode of action of the different physical and chemical agents may vary, they all cause damaging effects to the: (1) **cell wall** by lysis or inhibition of cell wall synthesis, (2) **cell membrane** by lysis or impairment of the mechanisms for taking up solutes, (3) **cytoplasm** by denaturing cellular proteins, (4) **enzymes** by inactivation or coagulation, and/or (5) **nucleic acids** by affecting their structure and function.

39

DETERMINATION OF THERMAL DEATH POINT and THERMAL DEATH TIME

In spite of the diversity among microorganisms as to their range of temperature tolerances, extremes in temperature can be used to control bacterial growth. Low temperatures, even those of lyophilization, will inactivate enzymes, producing a bacteriostatic effect, while high temperatures irreversibly denature cellular and enzymatic proteins and are, therefore, bactericidal.

The rate at which a bacterial population is killed by heat is determined by a number of factors: (1) the particular species; (2) the growth phase of the population; (3) the concentration of the population; (4) the presence of spores; (5) the exposure time; (6) the intensity of the heat; (7) the nature of the substrate; and (8) the presence of other control agents such as hydrogen ion concentration. It should be noted that these factors are also applicable to the control of bacteria by other physical and chemical agents.

In order to compare the relative susceptibility of different organisms to elevated temperatures it is necessary to determine their response under various conditions using precise scientific procedures. Two methods are routinely used: the **Thermal Death Point (TDP),** the lowest temperature at which a standard suspension of a given bacterial species is sterilized in ten minutes, and the **Thermal Death Time (TDT),** the shortest time required to sterilize the suspension of a certain species at a given temperature. Because of the number of other factors involved, the numerical values of TDP and TDT show some variability and are useful only as guidelines. These principles are the basis for the standard methods of microbial control in the preservation of food, pasteurization of milk, preparation of sterile media and hospital supplies, and decontamination of materials.

This experiment will determine the TDT of five (5) bacterial cultures: a Gram-positive cocci at high and low concentrations, a bacillus in the vegetative and spore-forming stages of growth, and a Gram-negative bacillus. By exposing each culture to heat of a specific intensity and taking samples at specified intervals, it is possible to determine the TDT of each culture. The determination of species differences may be made by comparing the TDT of the three different bacteria. The TDP will be determined by exposing each bacterium to a range of heat intensities for ten (10) minutes.

5. Repeat this process at exactly five (5) minutes, ten (10) minutes, fifteen (15) minutes, and thirty (30) minutes. Be sure to check the water bath frequently and maintain the temperature at 63°C.

6. Prepare a 100°C water bath. Transfer the thermometer still within the nutrient broth tube to the 100°C water bath or prepare another.

7. Place one tube of nutrient broth in the water bath and allow it to come to 100°C as indicated by the thermometer tube.

8. Remove 1 ml of the test organism and place it in the heated nutrient broth in the water bath. Note the time and record it. Discard the pipet. Set the culture sample aside.

9. After exactly 1 minute, remove a loopful of the heated culture and inoculate the 100°C; 1 min. tube. Return the broth culture to the bath immediately and place the inoculated tube in the rack.

10. Repeat this process at two (2) minutes, three (3) minutes, and five (5) minutes. Be sure to maintain the water bath t 100°C.

11. Incubate the inoculated tubes at 37°C until the next laboratory session.

B. Determination of Thermal Death Point

1. Label four (4) nutrient broth tubes with the name of the organism, and the growth stage or concentration if applicable. Label the exposure temperatures: 100°C; 90°C; 80°C; 70°C, and 60°C.

2. Place five (5) nutrient broth tubes in the water bath to be heating.

3. Transfer 1 ml of the test organism to one of the heated tubes in the water bath. Record the time and discard the pipet.

4. In exactly ten (10) minutes, transfer one loopful of the heated culture to the tube labeled 100°C.

5. Turn off the heat and cool the water to 90°C by slowly adding water and mixing after each addition. If you cool it too far, turn the heat on once more. Remove some of the water if necessary.

6. Repeat procedures 3 and 4, placing the inoculum in the 90°C tube.

7. Cool the water bath to 90°C and repeat the process. Continue, repeating the process at 70°C and 60°C, exposing the culture for only ten (10) minutes each time.

8. Incubate the inoculated tubes at 37°C until next laboratory session.

40

EFFECTS OF HYDROGEN ION (pH) CONCENTRATION ON GROWTH

With the exception of heat, hydrogen ion concentration exerts the greatest influence on bacterial growth. As with temperature, each species has its own range of tolerance. Those pH values above and below the range of the bacterium result in the inactivation of enzymes. While, as in the case of *Enterobacter aerogenes*, high acidity stimulates or induces the production of enzymes which tend to reduce the concentration of acids, there are limits of pH tolerances for all microorganisms.

Materials:

Broth cultures of:

Escherichia coli
Alcaligenes viscolactis
Staphylococcus aureus
Enterobacter aerogenes

Four (4) glucose agar deeps with phenol red (pH 3, 5, 7, and 9)
Four (4) Petri plates

Methods:

1. Work in teams of four (4) members, each member testing all the organisms at one pH value.
2. Divide the plates into four (4) sections and label each section with the name (initials) of one of the test organisms. Indicate the pH of the test medium.
3. Melt the glucose agar deeps and pour the plates.
4. Inoculate each of the organisms into the appropriate section by using a single streak of the loop.
5. Incubate at 21°C.
6. Examine for growth at 2 and 7 days and record your team results.

41
EFFECTS OF OSMOTIC PRESSURE IMBALANCE

The osmotic balance of water entering and leaving the bacterial cell is an important aspect of the growth and survival of bacteria. While some microorganisms tolerate high solute concentrations, as seen in some marine forms, most require an osmotic pressure just slightly lower than that of the cytoplasm.

Generally, and within limits, bacteria are able to survive in **hypotonic** solutions with solute concentrations considerably lower than that within the bacterial cytoplasm. The rigid cell wall tends to limit the amount of water that can enter the cell. However, when bacteria are placed in **hypertonic** solutions, growth may be seriously limited. Hypertonic solutions withdraw water from the cell causing the cytoplasm to become dehydrated and shrink away from the cell wall. Some bacteria are simply inhibited under these conditions and will return to normal when placed in an **isotonic** solution. Others may be irreversibly affected due to the permanent inactivation of enzymes.

The effect of osmotic pressure is of practical importance in food preservation. Such foods as jam, sauerkraut, condensed milk, salted meats and fish, owe their lasting qualities to desiccation of contaminating microorganisms by high osmotic pressure. Preservation does not mean foods are sterile. Many viable forms will grow when the osmotic pressure becomes more favorable.

Materials:

Broth cultures of:
- *Staphlococcus aureus*
- *Streptococcus liquefaciens*
- *Saccharomyces cervisiae* or *Halobacterium salinariura*
- *Aspergillus niger*

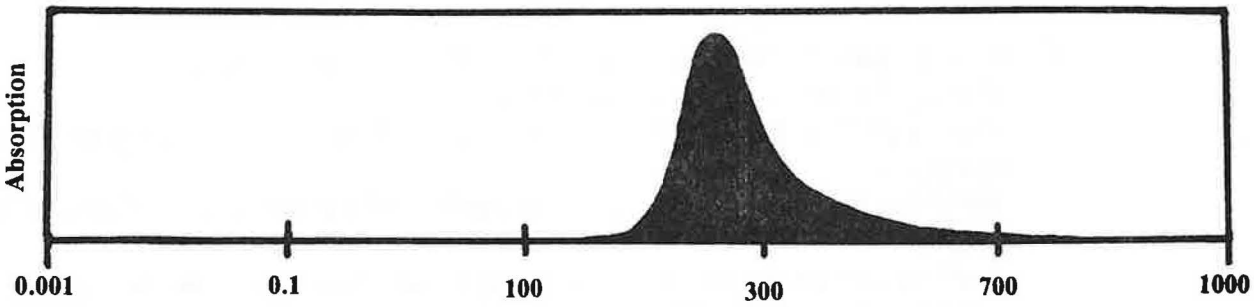
Raisins and raw hamburger
One (1) nutrient agar deep
Four (4) nutrient agar deeps with varying concentrations of NaCl (5, 10, 15, and 25%)
Three (3) nutrient broths with varying concentrations of NaCl (5, 15, and 25%)
Three (3) malt-extract broths with varying amounts of sucrose (15, 30, and 60%)
Five (5) Petri plates

42
EFFECTS OF ULTRAVIOLET RADIATION

U.V. light with wavelengths of 265 nm affect the chromosomal DNA of microorganisms by forming covalent bonds between adjacent thymine residues. These bonds distort the molecule and interfere with DNA transcription during protein synthesis and replication during cell division. Sublethal radiations can cause inheritable mutations by altering nucleotide sequences.

The bactericidal effect of exposure of a culture to U.V. radiations can be reduced somewhat by the immediate exposure to visible light with wavelengths between 365—450 nm. This reversal, called **photoreactivation**, results when visible light activates an enzyme, present in some microorganisms, that breaks the bonds caused by U.V. light, allowing the DNA molecule to resume its function.

Bactericidal activity of U.V. light: Absorption by nucleic acids



Short wavelengths of light are lethal and mutagenic to most microorganisms. This includes both the ionizing radiations of gamma and X-rays and the somewhat longer electromagnetic radiations of ultraviolet (U.V.) light. Because of its low penetration, U.V. light has very limited use for sterilization. Its primary value is in surface and air disinfection. The bactericidal effects of U.V. light are limited to a narrow portion of the U.V. spectrum where microbial nucleic acids, primarily DNA, exhibit the strongest absorption.

CHEMICAL AGENTS OF CONTROL

Many antispetics and disinfectants are available for the prevention of contamination and infection. They vary widely in their method and range of action and their practical use. The effectiveness of a disinfectant depends on a number of factors: (1) the nature and number of contaminating organisms, particularly spore-formers, (2) the concentration of the chemical, (3) the length of exposure to the chemical, (4) the amount of extraneous material such as soil, blood, saliva, etc., (5) the type of material to be disinfected, and (6) the temperature and pH of application. Therefore, the choice of an effective disinfectant is influenced by the purpose and the conditions of use.

Although many methods of evaluation of disinfectants are available, no single method or combination of methods has proved satisfactory. It has been proposed that a panel of testing procedures be used to provide more complete information about the various antiseptics and disinfectants under **conditions of use**. The increasing number of hospital-acquired *Staphylococcus* infections has stressed the need for a re-evaluation of the methods and materials used to control bacteria in the hospital environment.

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EVALUATION OF DISINFECTANTS AND ANTISEPTICS AND THE BACTERIOSTATIC ACTION OF CHEMICAL AGENTS

A widely-used method for evaluating the effectiveness of disinfectants and antispetics is the disk test. In this test, paper disks, saturated with various disinfectants, are placed on heavily inoculated agar plates. Following incubation, the plates are examined for zones of inhibition of bacterial growth around the disks. The presence of inhibition indicates the sensitivity of the bacterium to the disinfectant under the test conditions, rather than the degree of effectiveness of the chemical agent. Samples of the medium in the zones of inhibition are tested to determine bacteriostatic activity as opposed to bactericidal action.

Materials:

Broth cultures of:
- *Excherichia coli*
- *Bacillus cereus*
- *Staphylococcus aureus*
- *Mycobacterium smegmatis*

Four (4) nutrient agar deeps
Four (4) Petri plates
Samples of disinfectants and antiseptics
Four (4) sterile swabs
Sterile disks
Forceps
70% alcohol

Methods:

1. Work in teams of four (4) members using one prepared culture per member.
2. Prepare and dry an agar plate.
3. Inoculate the agar plate with your culture using the swab method for confluent growth. Allow it to dry for fifteen (15) minutes.
4. Place the agar plate over the sensitivity disk pattern. The pattern indicates the proper placement of the disks as described by standard methods.

44
EFFECTS OF DYES AND METALS

Many of the dyes not only stain bacteria but inhibit their growth in high dilutions. The basic dyes, crystal violet, gentian violet, and brilliant green, have a selective action against Gram-positive bacteria. Unfortunately, the dyes are readily absorbed and neutralized by serums and other proteins, thus limiting their use as selective agents in culture media and in the treatment of local lesions on the skin and in the mouth and vagina.

Numerous metals, such as silver, mercury, arsenic, copper, and lead, exert an oligodynamic effect on bacteria by poisoning enzyme activity. For example, organic compounds of mercury such as merthiolate, Metaphen, and mercurochrome, are relatively non-irritating and have been used as antiseptics for skin and mucous membranes. However, studies suggest that mercurochrome is not as effective in vivo as in vitro experiments indicate. This experiment will demonstrate the use of dyes and metals in the control of bacteria.

Materials:

Broth cultures of:
- *Bacillus cereus*
- *Enterobacter aerogenes*
- *Staphylococcus aureus*

One (1) MacConkey agar deep
Four (4) nutrient agar deeps
Sample of various metals
Four (4) Petri plates
Spatula
Forceps
70% alcohol

Methods:

Work in teams of four members. One member should prepare the study on dyes, while the others test the effects of metals on the different types of bacteria.

A. Dyes:

1. Melt one (1) MacConkey and one (1) nutrient agar deep.
2. Pour the MacConkey agar into a plate and allow it to solidify.
3. Sterilize the spatula by dipping it into the alcohol and igniting.
4. Aseptically, cut the solidified agar in the plate in half and remove one side.

45
INTERFERENCE OF DISINFECTANT ACTIVITY

The presence of organic matter or other materials such as lubricants, soaps, and detergents, inactivate many disinfectants and cause substances that typically show high activity to become inert. There are a number of ways in which these substances may alter the disinfectant activity: (1) by surface adsorption by protein colloids, (2) by the formation of inert or less active compounds, and (3) the active groups of foreign protein binding with the disinfectant.

In this experiment, inactivated yeast cells will represent the interfering organic matter. Several disinfectants will be tested against several representative types of bacteria in the presence and absence of organic matter.

Materials:

Broth cultures of:
- *Bacillus subtilis*
- *Escherichia coli*
- *Staphylococcus aureus*
- *Mycobacteria smegmatis*
- Inactivated yeast cells

One (1) tube sterile physiological saline
One (1) disinfectant (10 ml)
Eight (8) nutrient agar deeps
Eight (8) Petri plates
Two (2) sterile blanks
Two (2) pipets (1 ml)

Methods:

1. Work in teams of four (4) members with each member testing one of the prepared bacterial cultures.

2. Label one Petri plate **Control** and another **Experimental**. Divide the plates into four sections each. Label the sections **1 min.**, **5 min.**, **10 min.**, and **20 min.**

3. Melt the agar deeps and pour the plates.

4. Label two sterile blanks **Control** and **Experimental**.

5. Place 1 ml of sterile physiological saline in the Control tube and then transfer 1 ml of the inactivated yeast to the Experimental tube. To conserve pipets, let one person make these transfers. Transfer the saline first and then the yeasts.

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DETERMINATION OF THE EFFECTIVENESS OF MOUTH WASHES

An effective mouth wash should possess the characteristics of an ideal disinfectant: it should be highly toxic to microorganisms; non-toxic to human tissue; stable at room temperature and in air; have a pleasant odor and taste, and be inexpensive. As no disinfectant possesses all these properties, it is necessary to prepare a disinfectant that meets as many of these criteria as possible and still be acceptable to the buying public. This experiment is designed to test the relative effectiveness of various commercial mouth washes to sterile water.

Materials:

50 ml commercial mouth wash
Six (6) nutrient agar deeps
Six (6) Petri plates
Six (6) sterile water blanks (9.9 ml)
Three (3) sterile blanks
Three (3) pipets (1 ml)
One (1) paper cup

Methods:

1. Work in teams of two members. One team will use sterile water throughout the test and function as a **control** for the other teams which will test different commercially marketed mouth washes.

2. Melt the nutrient agar deeps and place in a 45°C water bath until ready to make the pour plates.

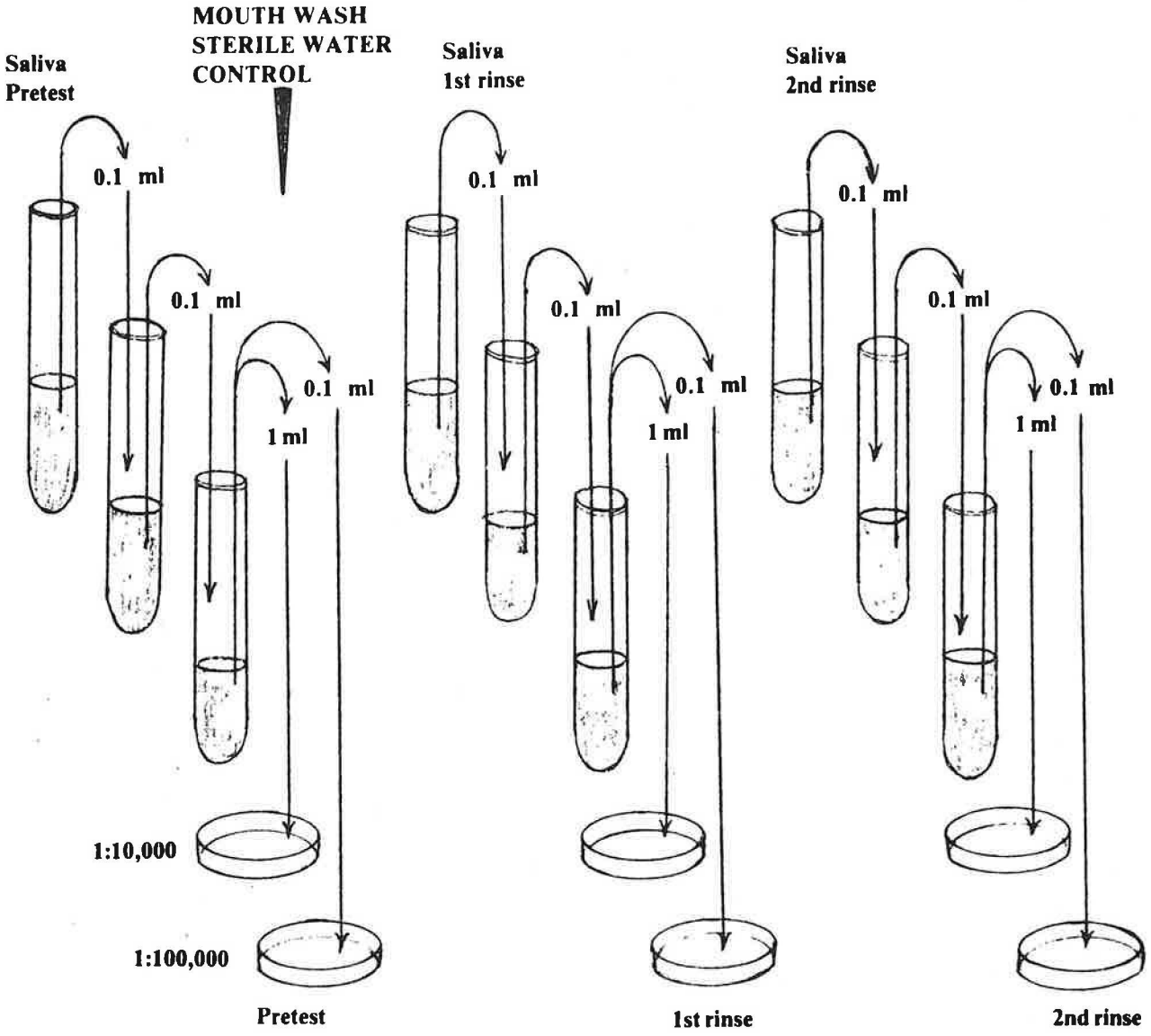
3. Place 4—5 ml of saliva in a test tube, replace the cap and shake the tube to mix well. Label this tube **pretest control.**

4. Rinse your mouth thoroughly with mouthwash or the sterile water if you are on the control team. Discharge this fluid into the sink.

5. Immediately rinse your mouth thoroughly with tap water and discharge this fluid into the sink.

6. Place 4—5 ml of saliva in another test tube and mix as before. Label this tube **first rinse.**

7. Remove 0.1 ml of saliva from the pretest control tube to a 9.9 ml water blank and mix by rolling the tube between the palms of your hands. This is a 1:100 dilution.


MOUTH WASH
STERILE WATER
CONTROL
Saliva
Pretest
Saliva
1st rinse
Saliva
2nd rinse
0.1 ml
0.1 ml
0.1 ml
1 ml
0.1 ml
0.1 ml
0.1 ml
1 ml
0.1 ml
0.1 ml
0.1 ml
1 ml
1:10,000
1:100,000
Pretest
1st rinse
2nd rinse

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ISOLATION OF ANTIBIOTICS FROM SOIL

Antibiotic substances are distributed widely throughout nature and play an important role in the regulation of microbial populations in soil, water, sewage, and compost. Several hundred antibiotics have been prepared in purified form but only a few of these have been sufficiently nontoxic to humans to be of any use medically. The majority of antibiotic agents that have been isolated fail to show the selective toxicity toward microorganisms necessary for the treatment of infectious diseases. Still, there remains a need for new and better antibiotics. No antibiotic is effective against all infectious organisms and there remain pathogenic organisms for which no effective antibiotic has been discovered.

In this experiment, an agar plate will be prepared using a soil sample dilution and a glucose nitrate-salts agar medium. This medium contains glucose as a source of energy, nitrate salts as the only source of nitrogen, and mycostatin to inhibit the growth of molds and is selective for the growth of the genus *Streptomyces* noted for the production of antibiotics. After incubation, those plates with surface growth of *Streptomyces sp.* will be overlaid with nutrient agar seeded with *Staphylococcus aureus* and incubated once more. The presence of clear zones around the *Streptomyces* colonies represents areas where *Staphylococcus aureus* was unable to grow due to the presence of an antibiotic.

Materials:

Broth culture of *Staphylococcus aureus*
Soil sample (0.5 gm)
Three (3) glucose nitrate-salts agar deeps (with mycostatin)
One (1) nutrient agar deep
One (1) nutrient agar short (4 ml)
Four (4) Petri plates
Sterile dilution blanks
Pipets (1 ml)

Methods:

1. Work in teams of two (2) members.
2. Melt the glucose nitrate-salts agar deeps and pour three (3) agar plates.
3. Weigh out 0.5 gm of your soil sample and, following your instructor's directions, prepare 1:5000, 1:10,000, and 1:100,000 dilutions.
4. Transfer 1 ml of each dilution to a plate and label the plates with the dilution factor.

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DISK DIFFUSION TEST OF ANTIBIOTICS

Most clinical laboratories now use the Standardized Disk Susceptibility Test to determine the effectiveness of antibiotics against infectious agents. This procedure provides reliable information not only of the choice of antibiotics but also the most effective concentration to use against a specific pathogenic organism isolated from a clinical specimen. In the test, the zone of inhibition produced by a single antibiotic-containing disk is measured and classified by referring to a standardized table as either resistant, susceptible, or intermediate. In the intermediate zone are those antibiotics to which the bacterium is not fully resistant or susceptible. Though other factors may enter into the choice of an antibiotic, such as possible side effects and patient allergies, this technique offers the physician precise information concerning most effective chemotherapeutic agent at the most effective dosage.

Materials:

Broth cultures of:

Escherichia coli

Staphylococcus aureus

Two (2) Mueller-Hinto agar deeps

Two (2) Petri plates

Two (2) sterile swabs

Antibiotic disks

Millimeter ruler

Methods:

1. Work in teams of two members, each using one of the cultures prepared as directed in **Section B. Preparation of Inoculum, Standardized Disk Susceptibility Test.**

2. Follow the directions as outlined in the standardized test.

3. After measuring the zones of inhibition, refer to the following chart to interpret your results.

Interpretation of Zone diameters in Disk Diffusion Technique

Antibiotic	Disk Content	Diameter (mm) of Zone of inhibition Resistant	Intermediate	Susceptible
Amikacin	30 ug	14 or less	15—16	17 or more
Ampicillin				
Gram-negative rods and enterococci	10 ug	11 or less	12—13	14 or more
Staphylococci and highly penicillin-sensitive organisms	10 ug	20 or less	21—28	29 or more
Bacitracin	10 units	8 or less	9—12	13 or more
Cephalordine	30 ug	11 or less	12—15	16 or more
Cephalothin	30 ug	14 or less	15—17	18 or more
Chloramphenicol	30 ug	12 or less	13—17	18 or more
Colistin	10 ug	8 or less	9—10	11 or more
Erythomycin	15 ug	13 or less	14—17	18 or more
Gentamicin	10 ug			13 or more
Kanamycin	30 ug	13 or less	14—17	18 or more
Methicillin	5 ug	9 or less	10—13	14 or more
Nafcillin and oxacillin	1 ug	10 or less	11—12	13 or more
Neomycin	30 ug	12 or less	13—16	17 or more
Novobiocin	30 ug	17 or less	18—21	22 or more
Nitrofurantoin	300 ug	14 or less	15—16	17 or more
Oleandomycin	15 ug	11 or less	12—16	17 or more
Penicillin G				
Staphylococci	10 units	20 or less	21—28	29 or more
Other organisms	10 units	11 or less	12—21	22 or more
Polymyxin B	300 units	8 or less	9—11	12 or more
Streptomycin	10 ug	11 or less	12—14	15 or more
Sulfonamides	300 ug	12 or less	13—16	17 or more
Tetracycline	30 ug	14 or less	15—18	19 or more
Vancomycin	30 ug	9 or less	10—11	12 or more

MICROBIOLOGY OF WATER

The bacteria indigenous to water are not typically of sanitary concern. However, due to the increased numbers of people, of technological development, and the related pollution of water by human and industrial wastes, most water available for municipal use is subject to contamination and may contain pathogenic organisms. Epidemiological evidence has established the relationship between waterborne disease and the presence of organisms of intestinal origin.

The organisms most widely used to indicate fecal contamination are the so-called *coliform group*, which by definition includes all the aerobic and facultative anaerobic, Gram-positive, nonspore-forming, rod-shaped bacteria that ferment lactose with the formation of gas within 48 hours at 35° C, and includes *Escherichia coli* and a number of closely related bacteria. Because of the almost universal presence of *Escherichia coli* in the human intestinal tract and because of the ease with which it can be identified and counted in a water sample, the presence of this bacteria is used as an indication of fecal pollution of water.

Two of the methods most commonly used to detect the presence of coliforms are the standard plate count and the membrane filter technique. The standard plate count is used to determine the effectiveness of water treatment measures by comparing the bacterial numbers before and after each step of the treatment.

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MOST PROBABLE NUMBER METHOD

The Most Probable Number (MPN) method is a multiple tube fermentation technique that consists of three parts:

1. **Presumptive Test.** Fermentation tubes of lactose broth are inoculated with aliquots of the water sample and incubated at 37°C for 24 to 48 hours. The presence of coliform bacteria is indicated by the presence of acid and gas. The Presumptive Test also makes it possible to obtain a statistical estimate of the number of organisms present in the water sample. The number of coliform bacteria present is based on the number of positive tubes found following incubation of the fermentation tubes as indicated on the **MPN Index Chart**.

2. **Confirmed Test.** EMB agar plates are streaked with an inoculum from the positive presumptive tubes and incubated at 37°C for 24 hours. The production of colonies with a green metallic sheen indicates the presence of *Escherichia coli.*

3. **Completed Test.** An isolated colony is transferred to a nutrient agar plate to perform a Gram stain and to a lactose fermentation tube and incubated at 37°C for 24 to 48 hours. The presence of acid and gas in the fermentation tube and a Gram-negative bacilli upon microscopic examination indicates a positive completed test.

Materials:

Water samples
Three (3) double strength lactose fermentation tubes
Seven (7) single strength lactose fermentation tubes
Pipets (10 ml and 1 ml)
One (1) EMB agar deep
One (1) nutrient agar deep
Two (2) Petri plates

Methods:

Work in teams of four (4) members with each team using a different water sample.

A. Presumptive Test

1. Melt the EMB and nutrient agar deeps and pour the plates. Set them aside for the confirmed test.
2. Shake the water sample to resuspend all material.

B. Confirmed Test

8. Streak the EMB agar plate for isolation using an inoculum from a positive presumptive tube.
9. Incubate at 37°C for 24 hours.
10. Examine the plate for the presence of colonies showing a green metallic sheen. Atypical colonies may be pink, mucoid colonies with dark centers.

C. Complete Test

11. Transfer an isolated colony on the EMB agar plate to the single strength lactose fermentation tube. Using the same colony, streak the nutrient agar plate.
12. Incubate at 37°C for 24 to 48 hours.
13. Examine the fermentation tube for evidence of acid and gas production. If the results are positive, make a Gram stain of one of the isolated colonies on the nutrient agar plate. Examine the slide for the presence of Gram-negative short bacilli. A positive completed test indicates the sampled water is not potable.

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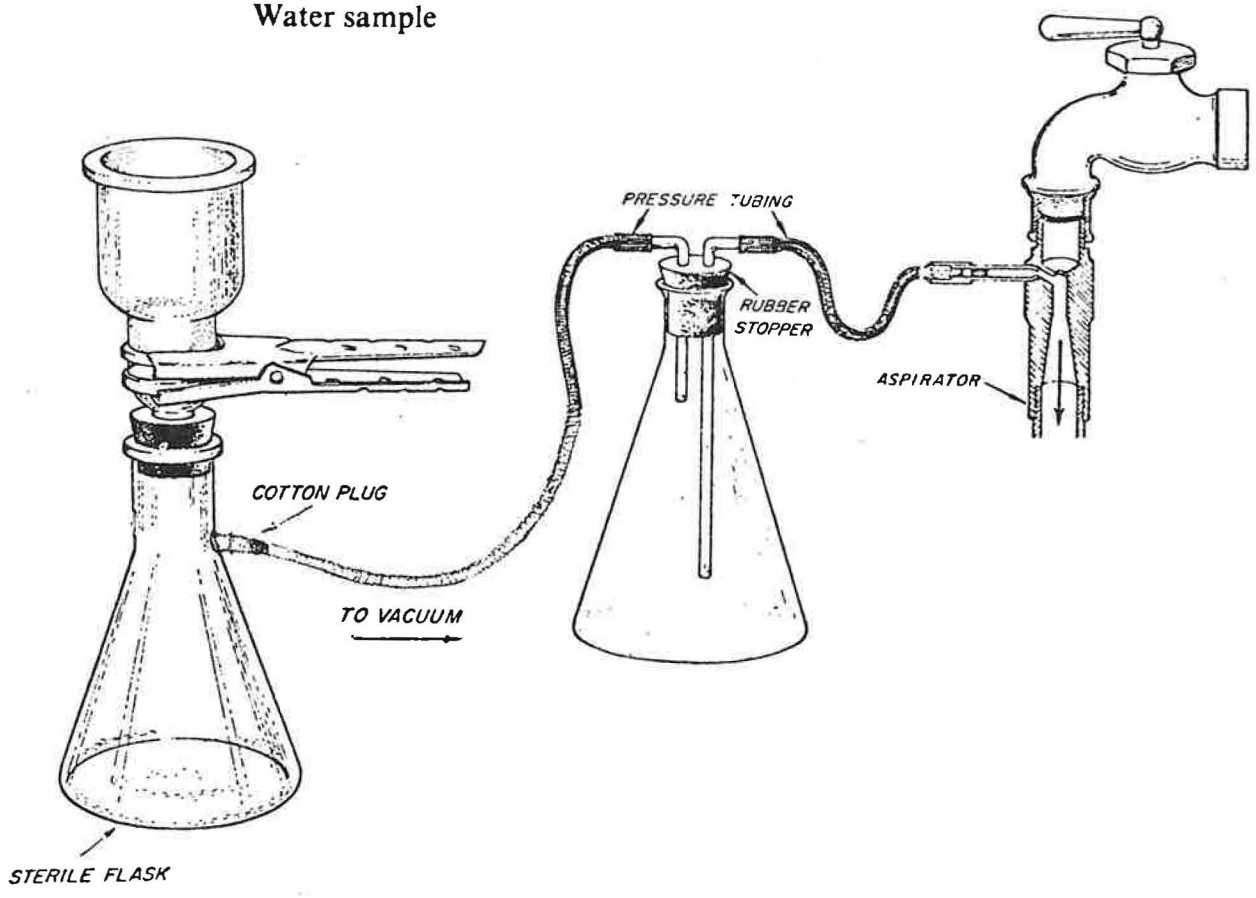
MEMBRANE FILTER TECHNIQUE

The membrane filter technique is routinely used in laboratories to analyze the bacterial load of water and other materials. In this technique, a sample or a dilution of a sample is filtered through a bacteriological filter with a pore size of 0.45 um. Bacteria larger than 0.47 um cannot pass through and are trapped on the membrane which is then placed on nutrient media for incubation and growth of colonies. This technique gives accurate counts if the colony count is between 50 and 200 organisms per filter disk. By placing the membrane on selective and differential media, the number of *coliforms* in a water sample may be determined. This technique has been recognized by the U.S. Public Health Service for the detection of *coliforms* in water.

Membrane-Filter Apparatus



6. Using sterile forceps, transfer a sterile membrane filter to the platform on the base of the filter unit, ruled side up.
7. Place the funnel over the base, making sure it is clamped securely.
8. Pour about 15 to 20 ml of sterile buffer solution into the funnel.
9. Shake the water sample to mix. Using a 10 ml pipet, transfer the volume to be sampled to the funnel.
10. Using the pipet, transfer an equal amount of buffered solution into the funnel twice to rinse off the pipet.
11. Turn on the aspirator and filter all the fluid into the flask.
12. While the aspirator is still on, add a volume of buffer solution to the funnel equal to the amount of the fluid filtered to rinse the sides of the funnel.
13. Repeat using a second rinse. Continue running the aspirator until the filter appears dry, about one minute.
14. Turn off the aspirator and remove the funnel.
15. Using sterile forceps, transfer the membrane filter onto the absorbent pad in the appropriate Petri dish.
16. Examine the endo broth plates for *coliform* colonies. Count the colonies that are pink to red with a golden green metallic sheen on the plate having between 50 to 80 *coliforms* and no more than 200 total colonies. Examine the nutrient broth plate. It should give a total viable count of the number of cells per ml.
18. Calculate the number of organisms per 100 ml of the sample water using the formula:

$$\textbf{Count per ml} = \frac{\textbf{Total number of colonies counted}}{\textbf{Number of ml of the sample tested}}$$

MICROBIOLOGY OF MILK AND FOODS

In nature, milk is transferred directly to the offspring with little or no possibility of contamination through exposure to the environment. Under artificial methods of collecting and handling, bacteria enter milk from practically everything it contacts. If pathogens are among those that enter, the consumer's health is jeopardized.

The control of the numbers and kinds of bacteria in dairy products is of real concern in the prevention of spoilage of milk and milk products, the transmission of infectious diseases, and in the manufacture of diary products. The problems associated with producing good milk and dairy products involve surveillance of the health of the herd and the personnel tending the herd as well as close attention to the details of sanitary plant operation.

Before mandatory federal inspections of food products, numerous diseases were transmitted by foods. However, there still remain problems associated with the transfer of pathogenic microorganisms by food handlers. Also, animals normally contain microorganisms pathogenic to humans. If the sanitation processes are not adequate or if the original microbial load was too high, they can cause illnesses. The methods used to determine the sanitary quality of foods is to measure the content of certain indicator organisms and the bacterial numbers in foods. As in water samples, the presence of *coliforms* indicates contamination with fecal matter. The presence of large numbers of organisms could mean a greater potential for the presence of pathogens.

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STANDARD PLATE COUNT FOR MILK

A total count of bacteria in milk, as well as in other foods, is the most reliable indication of the care with which the milk has been produced and handled. However, it does not reveal the presence of specific human pathogens. Protection against these organisms is handled by animal care and inoculations, human sanitary and health practices, and by pasteurization of the milk itself. The discovery of coliform bacteria such as *Escherichia coli* and related species always indicates unsanitary conditions in handling and is an index of the potential presence of pathogenic bacteria.

The standard plate count will be used to determine the number of viable bacteria present in various milk samples. The acceptable standards for milk and dairy products have been set by the U.S. Public Health Service and the California Bureau of Milk and Dairy Products which are considerably stricter. Standards for coliform counts have also been set. The coliform count will be made by inoculating a measured amount of milk samples on an EMB agar plate, counting the number of *Escherichia coli* colonies and calculating the number of coliforms per ml. Similar methods will be used to determine bacterial counts in various other foods.

Dairy Product	**Maximum Number per Milliliter or Gram (California)**
Grade A, prior to pasteurization	50,000 (750 *coliforms*)
Grade A, raw	10,000 (10 *coliforms*)
Grade A, pasteurized milk and milk products (except for cultured products like yogurt and buttermilk)	15,000 (10 *coliforms*)
Grade A, pasteurized cultured products	10 *coliforms*)
Ice cream	25,000 (10 *coliforms*)

Materials:

Raw milk
Pasteurized milk from vending machines
Ice cream
Reconstituted powdered milk
Four (4) nutrient agar deeps
One (1) EMB agar deep
Five (5) Petri plates
Pipets (1 ml)
Two (2) sterile 99 ml water blanks

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METHYLENE BLUE REDUCTION TEST

The reduction test is a simple procedure used to obtain useful but limited knowledge concerning the bacteriological quality of milk samples. Its primary value is as a screening test to survey a large number of samples at one time. In a milk sample that contains a large number of actively metabolizing microorganisms, the concentration of dissolved oxygen is rapidly reduced. Methylene blue loses its color in an anaerobic environment and is reduced. Therefore, the rate at which methylene blue is reduced following its addition to a milk sample indicates the relative quality of the milk.

Quality of Milk	Time Required for Reduction (Loss of Blue Color)
Excellent—Grade A	More than 8 hours
Good—Grade B	6½ to 7½ hours
Fair—Grade C	2½ to 6 hours
Poor—Grade C	Less than 2 hours

Materials

Raw milk
Pasteurized milk from vending machines
Ice cream
Reconstituted powdered milk
Five (5) pipets (10 ml)
One (1) pipet (1 ml)
Five (5) sterile blanks with caps
Methylene blue thiocyanate solution

Methods:

1. The class will be divided into two (2) teams, each team performing reduction tests on all the milk samples. The work may be divided into four parts: preparation of the tests and three subsequent checks at two-hour intervals. Select the time for the preparation of the test so that the final check is completed within the regular laboratory period.
2. Transfer 10 ml of each milk sample into labeled, sterile blanks.
3. Add 1 ml of methylene blue thiocyanate solution to each tube and invert the tube three (3) times **Do not shake the tubes**. This will aerate the milk and affect the reaction.
4. Record the time. Check every two (2) hours, noting the time that methylene blue is reduced to colorless form.

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STANDARD PLATE COUNT FOR FOODS

Though certain microorganisms are necessary in the production of foods such as yogurt, cheese, and pickles, the presence of others is of real concern in the spoilage of foods and as the causative agents of disease. Foods can serve as carriers of pathogenic organisms which cause such diseases as bacillary dysentary and cholera and as media in which pathogens may grow, some producing endotoxins that cause food infections when ingested and others secreting exotoxins that result in food poisoning.

In this experiment, the standard plate count will be used to determine the viable cell count and the presence of *coliform* bacteria, the indicators of fecal contamination.

Materials:

Various food samples
Three (3) nutrient agar deeps
One (1) EMB agar deep
Five (5) Petri plates
One (1) sterile 99 ml water blank
One (1) sterile 180 ml water blank
Pipets (1 ml)
Sterile sand or blender

Methods:

1. Work in teams of four (4) members. Each team will use a different food sample.
2. Melt the agar deeps and place the three nutrient agar deeps in the 45°C water bath.
3. Label the plates with the following dilution factors: **1:10, 1:100, 1:1000,** and **1:10,000.**
4. Pour the EMB agar plate using the plate labeled **1:10.**
5. Weigh out 20 grams of the food sample on sterile weighing paper.
6. If you are using the blender, place the weighed food sample and 180 ml of sterile water in the blender and blend for two (2) minutes. If you are using the sterile sand, add the weighed food sample and the sand to the 180 ml water blank and shake for three (3) minutes. These suspensions are 1:10 dilutions.
7. Allow the food particles to settle.

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SYNDER TEST FOR DENTAL CARIES SUSCEPTIBILITY

Dental decay is known to be initiated by the action of lactic acid, as well as other acids, which causes decalcification of tooth enamel. *Lactobacillis acidophilus, Streptococcus mutans*, and *Actinomyces odontolyticus*, among others in the oral flora, produce lactic acid from simple sugars. *Streptococcus mutans* produces exoenzymes that split the sucrose into fructose and glucose, forming long, insoluble, glucose polymers (glucan). In the process, fructose is made available for fermentation. The glucan polymers combine to form a network, called **dental plaque**, that holds the bacteria close to the teeth. The lactic acid bacteria ferment the fructose beneath the plaque causing decalcification and the initiation of dental caries.

The Synder test determines the amount of acid produced by oral flora and uses this as a measure of the susceptibility of an individual to dental caries. Saliva specimens are cultured on a medium containing glucose and pH indicator brom cresol green. This indicator is yellow at pH 4.4 and lower. Decalcification of dental enamel occurs in acid environments of pH 4.4 and less.

Assessment of Dental Caries Susceptibility			
Caries susceptibility	**Hours of Incubation**		
	24	**48**	**72**
Marked	Positive		
Moderate	Negative	Positive	
Slight	Negative	Negative	Positive
Negative	Negative	Negative	Negative

Materials:

Saliva specimen
Two (2) Synder test agar deeps
One (1) pipet (1 ml)
One (1) sterile blank (with cap)
Ice water bath (beaker with ice)

Methods:

1. Melt the Synder agar deeps and cool to 45°C.
2. Collect saliva over a three (3) minute period. Do not swallow.
3. Thoroughly shake the specimen tube and transfer 0.2 ml of saliva into one of the tubes. Mix by rolling the tube in the palms of your hands. Set the tube in the ice water to chill quickly.
4. Repeat step 3, using the other melted agar deep.
5. Incubate at 37°C for 72 hours, checking every 24 hours for evidence of acid production.
6. Using the table above to interpret your results, record your indicated caries susceptibility.

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COMPARISON OF SCRUB TECHNIQUES

Cleanliness is generally understood to mean **free from soil.** In food preparation and service, and certainly in the medical professions, its importance as a means of controlling the spread of disease is more clearly understood and appreciated. Since the studies by Semmelweis, it has become routine practice to wash hands prior to examining a patient and to do a complete surgical scrub prior to surgery. It requires about seven to eight minutes of washing with soap and water to remove all transient microorganisms. However, resident microorganisms are removed more slowly and are less susceptible than the transient microbes to the action of antiseptics.

Materials:

One (1) sterile water blank
Two (2) sterile swabs
Green soap and hand brush
Four (4) nutrient agar deeps
Four (4) Petri plates with watch glasses
Disinfectant
Sterile gauze pads

Methods:

1. Work in teams of two members. Designate yourselves **A** and **B**.
2. Melt the agar deeps, remove the Petri plate cover and aseptically pour just enough agar into each watch glass to fill it **nearly** full. Do not allow it to spill over or touch the top edge. Cover the plate and allow the agar to solidify undisturbed.
3. **A:** Moisten a swab in sterile water and rub it over an area on **B**'s forearm just a little larger than the diameter of the watch glass. Carefully remove the watch glass, place it, agar side down, over the swabbed area on **B**'s arm, place a gauze pad over it and tape it down securely. Allow it to remain in place for 15 minutes. Remove it and replace it in the Petri plate. Label the plate: **B-Control.**
4. **B:** Moisten a swab in disinfectant and swab **A**'s forearm. Allow it to dry. Continue as above. Label this plate: **A-Disinfectant.**
5. **B:** When your first test is completed, wash your forearm thoroughly with soap and water for one minute, rinse, strip, and air dry.
6. **A:** Place a watch glass, agar side down, next to the area first tested on **B**'s arm. Place a gauze pad over the watch glass and tape it down securely. Allow it to remain in place for 15 minutes. Remove it and replace it in the Petri plate. Label this plate: **B-1 minute wash.**
7. **A:** When your first test is completed, scrub your forearm with a hand brush, soap, and water for three (3) minutes, rinse, strip, and air dry.
8. **B:** Repeat step 6 by placing a watch glass on **A**'s forearm. Label this plate: **A-3 min. scrub.**
9. Incubate all four plates at 37°C until next laboratory session.
10. Examine the plates and evaluate the four methods of cleanliness.

SEROLOGICAL METHODS IN DIAGNOSIS

Since microorganisms are antigenic in nature and foreign to the human body, the body's immune system responds to their presence by producing specific antibodies that may eliminate these cells from the body. It follows that a patient's serum can be reacted with a known microbial antigen to determine, through antigen-antibody reactions, whether or not the patient has been in contact with the microorganism. Animals can be exposed to microbial antigens to stimulate antibody formation and their serum reacted with unknown antigens for identification. These concepts are the basis of serological tests which are an important part of diagnostic examinations.

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AGGLUTINATION REACTION

Agglutination tests involve an antigen-antibody reaction between particulate or cellular antigens and serum antibodies. Bacterial flagella, capsules, and cell components are able to stimulate antibody production which will result in the formation of visible aggregates of cells or particles when combined. An example is the hemagglutination of red blood cells.

In this experiment, the agglutination reaction will be used to identify an enteropathogenic bacterium causing acute gastroenteritis in children and will use somatic (O) and flagellar (H) antigens and antisera.

Materials:

Broth cultures of:
- *Escherichia coli*
- *Proteus vulgaris*
- *Enterobacter aerogenes*
- *Serratia marcescens*

Four (4) sterile physiological saline blanks
Four (4) sterile blanks
Pipet (1 ml)
Bacto-*Escherichia coli* OH and O antigens and antisera
Serological slide
70% alcohol

Methods:

A. Preparation of Heat-Killed Cultures

1. Work in teams of four (4) members, each member preparing one heat-killed culture.
2. Heat-kill a broth culture of a bacterium by placing the tube in a 100°C water bath for ten (10) minutes.
3. Transfer the heat-killed culture to a centrifuge tube and centrifuge the tube for five (5) minutes.
4. Decant the supernate into the original tube and place it in the receptacle for contaminated materials.
5. Suspend the centrifuged cells in 1 ml physiological saline and transfer to a sterile blank.
6. Label and refrigerate until next laboratory session.

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PRECIPITIN REACTION

Precipitin tests involve reactions between soluble antigens and serum antibodies which form complexes that precipiate out of solution as visible, fine granules. In the ring test, antiserum is introduced into a series of serological test tubes and the antigen carefully layered over it. After incubation of up to four hours, a ring of precipitate forms at the interface of the antigen and antibody in the tube having the optimum concentration.

Materials:

Physiological saline
Bovine globulin antiserum
Bovine serum dilutions of 1:25, 1:50, and 1:75
Pipets (0.5 ml)
Four (4) serological test tubes and rack

Methods:

1. Work in teams of two (2) members.
2. Label three (3) serological test tubes according to the antigen dilution: **1:25, 1:50,** and **1:75**. Label the fourth tube as a **saline control**.
3. Using a different pipet for each dilution, transfer 0.3 ml of each of the bovine serum dilutions into the appropriately labeled tube.
4. Transfer 0.3 ml of physiological saline into the control tube.
5. Carefully overlay 0.3 ml of the bovine globulin antiserum into each of the four test tubes.
6. Incubate at 37°C for 30 minutes.
7. Examine the tubes for a ring of precipitate at the interface of the antigen and antibody. Which tube showed optimal antigen:antibody ratio?

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MEDICAL-LEGAL APPLICATIONS OF SEROLOGICAL REACTIONS

The specificity of many antigenic proteins and the sensitivity of such serological reactions as the precipitin test make it possible to identify the species source of blood and tissue samples. Serological reactions are commonly used in the detection and identifiction of blood stains and adulterants of meat products.

IDENTIFICATION OF AN UNKNOWN STAIN

Before a suspected stain is tested serologically, it is necessary to determine whether it is blood or some non-biological material. Saline extracts of the sample are mixed with glacial acetic acid, hydrogen peroxide, and benzidine. If the appropriate blood or tissue enzymes are present. The peroxide will be broken down. The oxygen thus released oxidizes benzidine to a delicate blue-green color. This is **presumptive** evidence of blood as these enzymes are also present in materials other than blood.

Materials:

Samples of stained and unstained cloth
Four (4) test tubes (13 x 75 ml)
Physiological saline (5 ml per sample)
Glacial acetic acid (2 ml per sample)
Benzidine powder
Hydrogen peroxide, 3% solution (20 drops per sample)

Methods:

Work in teams of two (2) members throughout the total experiment.

A. Extraction of Stain Material

1. Thoroughly clean and rinse tubes with distilled water. These tests are very sensitive to extraneous materials.
2. Label two sets of two (2) tubes: **Unknown** and **Control**.
3. Pipet 5 ml of fresh physiological saline into one set of tubes.
4. Place a 2—4 cm^2 sample of the stained cloth (**Unknown**) and the unstained cloth (**Control**) into the appropriately labeled tubes.
5. Place both samples in the refrigerator and allow about 48 hours for extraction.
6. The extracts of the stained and unstained pieces of cloth will be used for the following tests:

B. Presumptive Test of Stain Material

1. Pipet 2 ml of glacial acetic acid in the second set of tubes.
2. Using a toothpick as a spatula, add a single scoop of benzidine power to each tube.

4. Incubate for one hour at room temperature,making observations every fifteen (15) minutes.
5. Record your results. If all tests with the unknown stain sample and known antisera are negative, the sample is either some other kind of blood or is not blood at all.

DETERMINATION OF UNKNOWN HUMAN BLOOD TYPE

The blood cells of humans can be differentiated into four types depending on the presence or absence of two chemical substances called A and B. These substances, which are called **agglutinogens**, are antigenic in nature and are located on the surface of the red blood cells. Blood cells having only the A substance are type A, blood cells having only the B substance are type B, blood cells having both A and B substances are type AB, and blood cells with neither A nor B are type O.

Landsteiner found that the blood serum of type A individuals contains natural antibodies which cause the blood cells having B agglutinogens to clump together. Likewise the blood serum of a type B person contains an antibody which will agglutinate the blood cells having A agglutinogens. The serum of an O individual will agglutinate cells of types A, B, and AB, but the serum of a type AB person will not agglutinate the cells of any of the other three groups. This reciprocal relationship is summarized in the following table:

Blood Type	Agglutinogen on Cells	Agglutinin in Serum
A	A	anti-B
B	B	anti-A
AB	A and B	neither
O	neither	anti-A and anti-B

Since the human A and B blood antigens are carbohydrate in nature, they are more stable than proteins and can be identified years later providing the sample has remained dry. Saline extracts of an unknown stain are mixed with standard amounts of known antisera, incubated, and retested against known A and B blood cells. If the agglutinating capabilities of the antisera has been inhibited, it is concluded that the sample contains specific A or B substances. Controls with the unstained sample must be made. The absence of A inhibition, especially when B is present, does not mean A was absent since blood types A_2 and A_3 are difficult to detect by this method.

Materials:

Sample of blood-stained cloth previously prepared by students (2 cm^2)
Sample of unstained cloth (2 cm^2)
Four (4) test tubes 13 x 75
0.5 ml anti-A serum 1:20 dilution per sample
0.5 ml anti-B serum 1:20 dilution per sample
0.5 ml 2% suspensions of A and B cells per sample
One (1) agglutination slide

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FUNGI

Fungi are among the most plentiful forms of life and are found in most environments, usually in the soil. Most are saprophytic and indispensable in the decomposition of plant and animal material. Economically, they are important in the production of acids, alcohol, and antibiotics. Though most are saprophytic, a few are parasitic. Fungi frequently cause plant diseases, but of the thousands of known species, less than 100 are capable of invading man or animals and less than a dozen produce fatal infections. The diseases produced by pathogenic fungi resemble those caused by *Mycobacterium tuberculosis* in that they develop slowly and cause chronic infections. Fungi do not produce toxins but cause hypersensitivity reactions and lesions such as tissue necrosis and abscess formation.

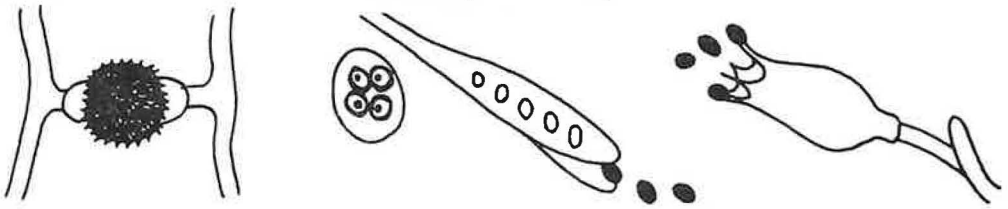
Clinical specimens containing pathogenic fungi are rarely seen. However, with the increased use of transplants and the need for immunosupressive drugs, the number of fungal infections in hospitals is rising. Rapid identification of clinical fungal isolates is necessary in that fungal infections, particularly the systemic type, are difficult to treat and invariably are fatal if not treated promptly.

Fungi are plants that lack chlorophyll, roots, stems, and leaves. The two major groups of fungi are the molds, which are multicellular, and the yeasts, which are unicellular. The individual filaments of molds are called **hyphae** (singular—hypha). If the filament has crosswalls, it is **septate**. A filament without crosswalls is **non**-septate. The mat of branching, intertwined hyphae is known as a **mycelium** and the entire plant is called a **thallus**. The mass of hyphae on and in the medium are **vegetative mycelium** and are involved in the assimilation of food. The hyphae that rise above the thallus are called **aerial mycelium**. The aerial mycelium may be involved in spore formation and therefore are called **fertile mycellium**. Fungi are placed in four (4) classes, Phycomycetes, Ascomycetes, Deuteromycetes, and Basidomycetes.

Sexual and asexual spores may be produced. Sexual spores are produced by the fusion of two nuclei from separate plants. Several types are formed:

- **Acospores.** The sexual spores of Ascomycetes are formed within a membrane or sac.
- **Basidiospores.** The sexual spores of Basidiomycetes are formed on a specialized structure, the basidium.
- **Zygospores.** Phycomycetes produce free spores, an example of one is the zygospore of **Rhizopus**. Another example are the oospores of **Saprolegnia**.

Sexual spores of fungi



Zygospore **Acospores** **Basidiospores**

Materials:

Sabouraud agar plates of:

Rhizopus nigricans
Aspergillus niger
Penicillum notatum
Mucor sp.

Slant culture of *Streptomyces griseus*

Broth culture of *Saccharomyces cerevisiae*

Prepared slides of:

Candida albicans
Mycobacterium tuberculosis
Mycobacterium leprae
Penicillium notatum
Rhizopus with zygospores
Aspergillus niger
Mucor sp.

Methods:

1. Examine the sabouraud agar plates of selected fungi for distinctive macroscopic characteristics of each culture.
2. Examine the prepared slides and note distinctive microscopic characteristics. The following structures should be identified on these species:
   - *Aspergillus niger*—conidiophores, conidiospores, sterigmata, and septate hyphae.
   - *Rhizopus nigricans*—sporangiophores, sporangiospores, sporangia, columella, non-septate hyphae, and rhizoids.
   - *Penicillium notatum*—conidiophores, conidiospores, sterigmata, and septate hyphae.
   - *Mucor sp.*—sporangiophores, sporangiospores, sporangia, columella, non-septate hyphae, absence of rhizoids.
3. Record your observations as drawings.
4. Prepare a wet mount of *Saccharomyces cerevisiae* and observe budding in the species. Add a loopful of a dye such as methylene blue.
5. Examine *Streptomyces griseus* either with low power on the compound microscope or with the dissecting microscope.

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PROTOZOA

The protozoa are unicellular, eucaryotic, heterotrophic organisms. They have a specialized cell more complex than the bacterial cell and the average cell of a multicellular organism. They have organelles to carry out such functions as nutrition, locomotion, respiration, excretion, and attachment. Most are microscopic or just barely visible to the unaided eye. As a rule, those that are parasitic are smaller than those that are not. The protozoa are primarily asexual, reproducing by binary fission, though some groups reproduce sexually. When subjected to adverse conditions, some strains form resistant cysts. These are important in the transmission of protozoan infections. When suitable conditions for growth recur, the cyst takes in water and the protozoan becomes active once more.

All protozoa possess some type of motility during some part of their life. Their classification is based on their means of locomotion, morphology, mode of reproduction, and type of nutrition. However, the means of locomotion is the major criterion. The four classes (or subphyla) of protozoa contain important parasites of man.

Materials:

Prepared slides of selected protozoa
Live cultures of:
Paramecium caudata
Amoeba proteus
Pond water specimens

Methods:

1. Examine the prepared slides of the selected parasitic protozoa.

2. Complete the chart on the following page indicating the location of these protozoa within the body and the method of diagnosis. Many of these protozoa are important health problems still facing the medical field.

3. Examine the prepared slides of *Paramecium caudatum*. Note the specializations including sexual reproduction. Prepare a wet mount and observe motility and feeding.

4. Examine the prepared slides of *Amoeba proteus* and prepare a wet mount to observe ameboid movement.

5. Examine the wild culture of protozoa in pond water.

6. Make sketches and record your observations in your log book.

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HELMINTHES

Collectively, the parasitic worms are called *Helminthes*. The species of medical and public health concern belong to two phyla: *Platyhelminthes* or flatworms which include the two classes of *Trematoda* (flukes) and *Cestoda* (tapeworms) and *Nematoda* or round worms which also includes two classes, the *Nematoda* (hookworms and pinworms) and *Annelida* (leeches). Most parasitic worms have complex life cycles involving one or more intermediate hosts before the definitive host is reached. As many as 30% of the population of the United States are infected by parasitic worms, but in most cases remain asymptomatic. In underdeveloped countries or where sanitary conditions are poor or lacking, large numbers of people have overt symptoms. Laboratory diagnosis relies on the isolation and identification of either eggs or larvae from the infected individual.

Materials:

Prepared slides and mounts
Charts of selected Heliminthes life cycles

Methods:

1. Examine the prepared slides and mounts.
2. Complete the chart on the following page indicating the location of each species of Heliminthes in the body and the method of diagnosis.
3. Examine charts and references to determine the life cycles of the assigned Heliminthes.
4. Make sketches and record your observations in your log book.