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ABSTRACT

This book was written to help introductory biology teachers gain a basic understanding of contemporary bioinstrumentation and the uses to which it is put in the laboratory. It includes topics that are most basic to understanding the nature of biology. The book is divided into five sections: (1) "Separation and Identification" that includes chapters on electrophoresis, chromatographic techniques, immunologic methods, flow cytometry, and centrifugation of biomolecules; (2) "Observation" that includes chapters on advances in light microscopy, transmission electron microscopy, and scanning electron microscopy; (3) "Spectroscopy" that includes chapters on absorption spectroscopy, fluorescence spectroscopy, cross-sectional medical imaging, and infrared spectroscopy; (4) "Biological Tracing and Sensing" that includes a chapter on radionuclides; and (5) "Manipulation of Biological Molecules" that includes chapters on recombinant DNA, the polymerase chain reaction, and restriction fragment length polymorphisms. Chapter overviews, concept maps, margin notes, photos of real scientists and their students, overhead transparency masters, and an Internet bioinstrumentation web site directory are also included. (JRH)

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BIOINSTRUMENTATION



Tools for Understanding Life

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BIOINSTRUMENTATION

Tools for Understanding Life

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Cover photograph: Chapter author, Lynda Britton, Assistant Professor of Medical Technology at Louisiana State University Medical Center, Shreveport, LA, instructs senior medical technology student, Stephanie Mason, on how to perform the polymerase chain reaction (PCR) using a thermal cycler.

Cover photograph by Stan Carpenter — Department of Medical Communications, LSUMC

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“To my Ph.D. students in Biology Education at LSU, past and present — Eleanor Abrams, John Trowbridge, Connie Nobles, Susan Nichols, Phyllis Griffard, Susan Tittlebaum, Mary Gail Yates, Lynda Britton, Sharon Flanagan, Knight Roddy, Kodie Jeffery, Dilek Buchholz, Jessica Scott, James Njengere, Judith Poindexter, Juliana Hinton, Jodale Ales, Marcella Hackney, and Dennis Wissing.”

JHW

“To John T. Jobe and Betty W. Johnson, who became my friends; Jimmy M. Cairo and Jim H. Wandersee, who became my mentors; and, above all, to my loving and supportive wife, Vicki, who stands with me at all times.”

DRW

“Early and mid-career science educators who are formally trained often find that they need self-education to help them understand contemporary advances in their discipline. To that end, and to the late Edward H. Birkenmeier, M.D., the second edition of *Bioinstrumentation* is dedicated.”

CTL

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Preface

James H. Wandersee

I am deeply aware of the importance of a new or improved instrument for progress in understanding life. Over the years I have devoured every source I could find in the history of biology about the “Father of Microbiology,” Antony van Leeuwenhoek (1632-1723). In fact, my very first NABT convention presentation (1980) was based upon his wonderful instruments and what he saw with them. Since then, I have spoken to audiences of biology teachers across the U.S. and abroad about this amateur biologist, telling the tales of his amazing discoveries. Oh, the instruments he made and the applications he found for them!

At a time when compound microscope lens systems were plagued by both spherical and chromatic aberration, this Dutch linen draper realized he could probably accomplish more with a single lens, by improving a common (toy) magnifier, the flea glass. Leeuwenhoek built and used 247 high quality, single-lens microscopes -- grinding 419 lenses, some as small as a pinhead. Not only did he build these microscopes entirely by himself, he developed methods (dark-field microscopy, microtomy, micrometry, three-axis mechanical stages, etc.) for their use that allowed him to see what no other person had ever seen before -- living protozoa, the eye of a louse, human red blood cells, *Spirogyra*, frog sperm, bacteria in human tooth scrapings, and so forth. People came from all over the world just to get a glimpse through one of Antony’s instruments. His microscope extended his vision (and ours) into the microcosmos. Although technology ultimately improved the compound microscope and made single-lens microscopes obsolete for biological research, it took almost a century for it to equal what Leeuwenhoek’s lenses could reveal. Even as late as 1831, the Scottish botanist, Robert Brown, of “Brownian motion” fame, discovered and named the *nucleus* (Latin for “little nut”) as a regular feature of cells -- using a single-lens microscope.

What do Leeuwenhoek’s microscopes have to do with contemporary bioinstrumentation? His technological improvements illustrate this simple fact: no amount of learning can overcome the limits of our sense organs. Sometimes biologists just have to wait to study a biological object or event until a new or improved instrument, method, or technique extends their sensory reach. Until that time, all they can do is push the current technology to its limits. Sometimes, though, that’s actually enough, for it allows them to continue to make progress in the interim.

In a recent interview study of life scientists at the university level, our research group found that *instrumentation* and *methods* drive research even more than *theory* does. Almost every laboratory wants to use the latest instruments and the newest methods; national funding agencies see the equipping of laboratories with state-of-the-art instrumentation as the prime way to improve scientists’ competitiveness and produc-

tivity. Yet, the instrument is but a tool for problem solving; one that supplements, but does not replace, a well-reasoned line of research and insightful integration of new knowledge with existing knowledge. Understanding the theory of the instrument -- how it does what it claims to do, its assumptions, limitations, and idiosyncrasies -- allows the expert to filter the results, discarding some and trusting others. To paraphrase (and bowdlerize) poet Alexander Pope, a little learning and a complex instrument (“black box”) are a dangerous thing!

This book was written to help introductory biology teachers gain a basic understanding of contemporary bioinstrumentation and the uses to which it is put in the laboratory. It has not escaped us that the book may have other learning applications, but high school biology teachers were the audience we planned to, and did, address.

After an extensive survey of major university professors who were actively pursuing biological and medical research, we chose the 16 topics to be included in this edition. Virtually all of the first edition’s content was changed or updated on the basis of this peer review process. There are so many laboratory instruments (and permutations of them) in use in the life sciences that we are sure “kibitzers” will find some they think we should have included. (Why no mass spectrometer? What about biosensors? Okay, we admit we might have done the same thing!) However, we ultimately included those that the university professors we surveyed thought were most basic to understanding the nature of biology -- not the most exotic or the most expensive or the most “cutting-edge.” We did not aim to be encyclopedic, but rather synoptic. We pass the baton to others for the former.

The science education reform movement informs us that it is as important for students to know “*how* we know something in biology” as it is to know “*what* we know.” Do you know how scientists use infrared (IR) spectroscopy to identify and determine the molecular structure of a biomolecule? Do you know how the polymerase chain reaction (PCR) “amplifies” DNA? Do you know how a flow cytometer sorts cells at a high rate of speed? Do you know how an ultracentrifuge can be used to separate macromolecules? Read this book and find out. The more *you* understand “how we know what we know in biology,” the more *your students* will. Plus, there’s the fun of your own “aha! experience” and the satisfaction of learning even more about your favorite subject -- biology.

In the American Association for the Advancement of Science’s *Benchmarks for Science Literacy* (1993, p. 47), we read that upon completing grades 9-12, students should understand “... the importance of technology to science ...” and that “... new technologies make it possible for scientists to extend their research in new ways or undertake entirely new lines of research” and that “the very availability of new tech-

nology itself often sparks scientific advances.” We assure you that all of those benchmarks can be pursued using knowledge gained by reading this book. We see this book as potentially helpful in attaining reform goals.

One last thing -- as teachers writing for teachers, we have included chapter overviews, concept maps, margin notes, photos of real scientists and their students, overhead transparency masters, and an Internet bioinstrumentation web site directory. We have edited our authors' explanations of the instruments for clarity, but we didn't water down the language of science. You may find some sections to be more challenging than others -- depending on your biology background and experience. We can assure you, however, that all chapters were written by persons who teach and reviewed by practicing biology teachers. The 1994 *NABT Mission Statement -- An Elaboration* states "... teachers must ... be treated professionally in all circumstances." As former and current biology teachers, we have attempted to do just that.

ACKNOWLEDGMENTS

I am grateful to Professor C. T. Lange, the distinguished editor of the 1988 first edition (called simply *Bioinstrumentation*) and well known to NABT and BSCS associates, for inviting me to undertake the principal editorship of a new edition and for giving me the freedom to modify it. His support and encouragement along the way provided inspiration. In addition, Professor Dennis Wissing served with great distinction in every stage of production, from coordinating the work among our many authors to tracking and transforming the manuscript from an idea to reality. He was involved in every major decision about the book, and I am deeply appreciative of all his efforts. Thanks are also due to Wayne Carley, NABT Executive Director; to Chris Chantry, Sherry Grimm, and Michele Bedsaul of the NABT Publications Department; and to Professor Dick Storey (Biology Department Chair, Colorado College) of the NABT Publications Committee for their help and advice. Finally, in the buck-stopping words of a previous NABT editor, William F. McComas (1994), "I alone take full responsibility for the editorial decisions that may have resulted in changes in meaning or style ... [when the chapters were] molded into the final common format." JHW

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Foreword

Over the last four decades, two key factors have spurred the growth of bioinstrumentation: rapid advances in technology and demands by scientists to observe, measure, and manipulate biological structures. Teams of biologists, chemists, engineers, physicists, and computer programmers have repeatedly met the challenges of developing and adapting technology to automation. The bioinstrumentation described in this book illustrates the merging of biotechnology and automation.

Past experiences in developing bioinstrumentation are indeed prologue to the future. In the 1950s, classic techniques of quantitative analytical biochemistry formed the basis of instrumentation. Simple photometers recorded percent transmission of light through a substance to measure analytcs. Rigid conformance to protocol and pipetting or measuring skills were as crucial as the instrumentation to the quality of results. In the 1960s and 1970s, cost effectiveness and ease of training to ensure technology transfer became important goals for evolving instrumentation. The latter 1970s heralded the beginning of computer-driven instrumentation and robotics to support operator adherence to protocols and to replace some of the manual support techniques, like pipetting. The growth that continued through the 1980s has led to an era of consolidation and retrenchment by developers of bioinstrumentation. Because of the significant investment and long years of development before new bioinstrumentation is marketable, the financial impact is carefully assessed before a product is launched.

Bioinstrumentation of the future will increasingly require an integration of the sciences and engineering. Noninvasive technologies such as biosensors, which do not require disruption of the organism during observation, measuring, and manipulation, will be developed. Computer interface with instrumentation will increasingly assist the user. Precision and accuracy of analyses will continue to be hallmarks for quality in bioinstrumentation, but cost and technology transfer will justifiably be forces in determining the instrumentation of the future.

The editors of this book have amassed a cadre of very talented authors, experts in specific areas of bioinstrumentation. The book is appropriately divided into categories of biotechnology and instrumentation. These are readily viewed as *core technologies*. For the user of this text, this is an important premise since adaptations of current bioinstrumentation, as well as the evolution of next generations of bioinstrumentation, will be based on an *understanding* of these core technologies.

John R. Snyder, Ph.D.

Bioinstrumentation: Tools for Understanding Life

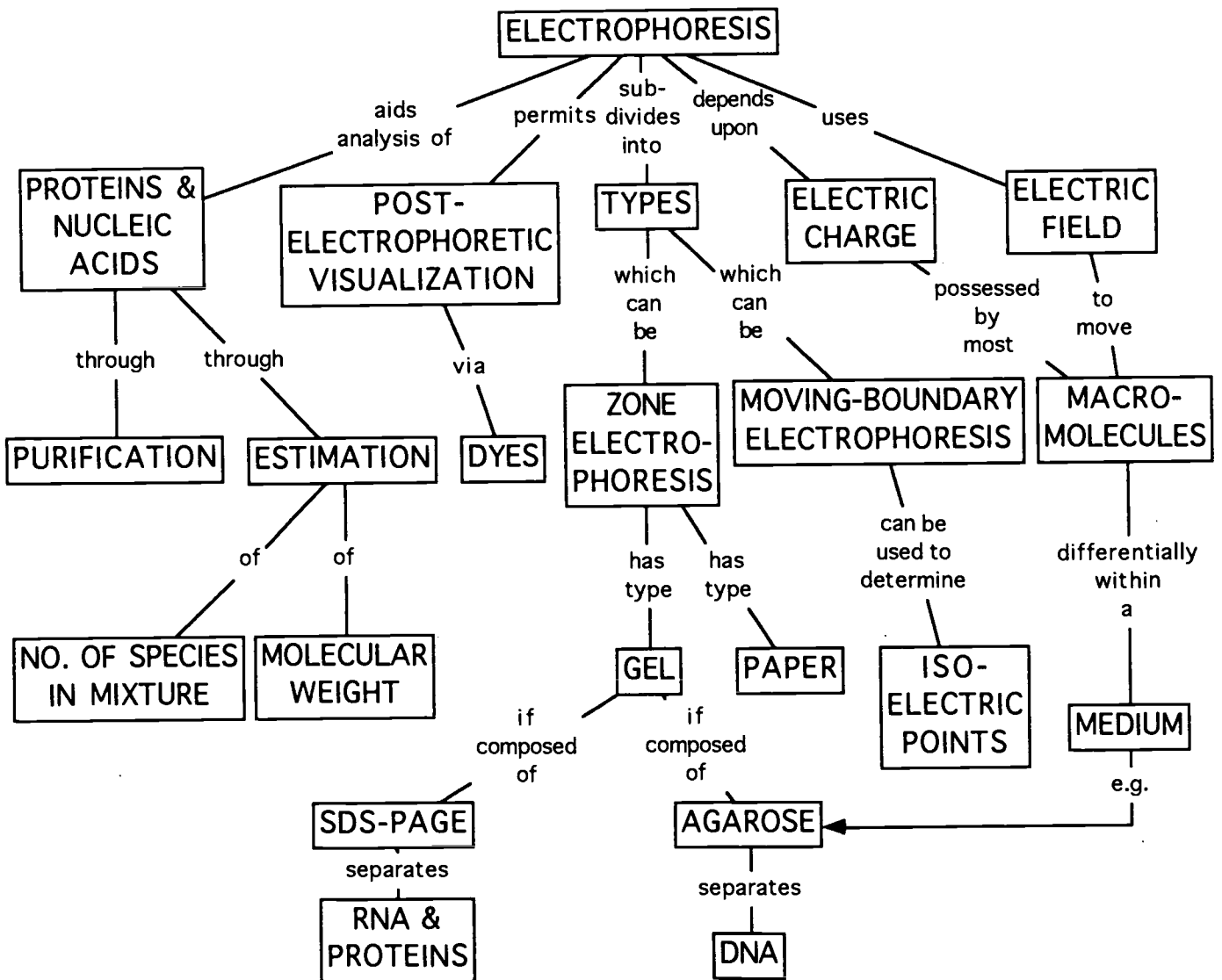
ELECTROPHORESIS

1

Terry S. LeGrand and Tak Yee Aw

Overview

1. Principle of electrophoresis
2. Types of electrophoresis
3. Protein electrophoresis
4. Agarose gel electrophoresis of double-stranded DNA



Electrophoresis

Electrophoresis is the movement of charged particles in an electric field.

Electrophoresis is derived from the Greek words *elektron*, beaming sun, and *phoresis*, the act of carrying. Thus electrophoresis is the “carrying” or movement of a charged particle by an electromotive force. Electrophoresis is a technique utilized in research and in the clinical setting for separation of proteins, isoenzymes, lipoproteins, transport proteins, and nucleotides in various body fluids. It is especially useful as an analytical tool, permitting visualization of proteins and nucleotides after electrophoresis by treatment with dyes, as well as estimation of the number of proteins in a mixture. Moreover, electrophoresis is used to determine the degree of purity of an isolated protein, and properties such as protein molecular weight. Other applications include determination of the nucleotide sequence of large DNA molecules and analysis of the structure of chromatin, the diffuse chromosomal material seen in the nucleus of non-dividing eukaryotic cells. Electrophoresis for separating proteins in solution was introduced in 1933 by Tiselius. By the early 1970s, it was possible to determine the length and purity of large DNA molecules using gel electrophoresis. Today, it is a widely used, innovative method for the analysis of body fluids and tissues.

Electrophoresis is used to separate proteins and nucleic acids.

Proteins have a net positive or negative charge, depending on the types of amino acids that make them up, and each nucleotide of DNA carries a single negative charge. Therefore, if proteins or DNA fragments are suspended in a solution, and an electric field is applied, the proteins or DNA fragments will move at a rate dependent upon their charge, size, and shape. Electrophoresis is a valuable tool because most macromolecules — the biological machinery present in every cell — are electrically charged and will therefore move within an electric field. This property can be utilized to distinguish between molecules of different charge and shape and to separate various kinds of macromolecules.

The Principle of Electrophoresis

The basic principle of electrophoresis is that a charged particle in a supporting medium will move toward an electrode with the opposite charge when an electrical field is applied (see Figure 1-1). Once the electric force is applied, an opposing force is produced by the viscous drag of the medium. A charged particle suspended in a medium will move in an electrical field (the applied force) at a constant velocity.

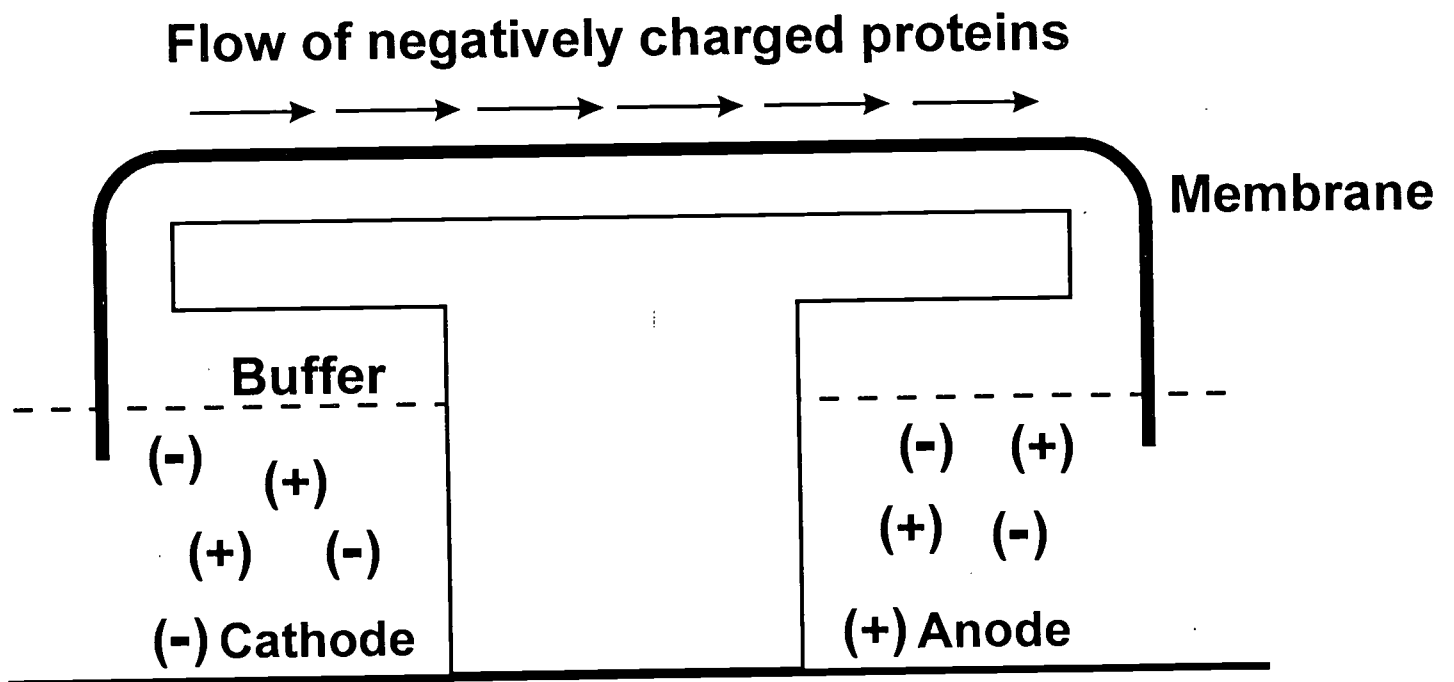


Figure 1-1

The principle of electrophoresis. Negatively charged proteins move toward the positively charged anode when an electric field is applied.

4 Electrophoresis

Particle velocity is a product of electrical force and viscous drag.

This velocity is determined by a balance between the electrical force that tends to move the particle and viscous drag or the resistance to the particle's movement provided by the medium. Mathematically, this principle may be stated as:

$$Eq = fv$$

where E represents the electric field, q is the charge on the suspended particle, v is the velocity, and f is the frictional coefficient. Thus fv represents viscous drag. Mobility is dependent upon the frictional coefficient, which is a function of physical characteristics of the molecule (such as size and shape), properties of the medium, and the electric field.

Types of Electrophoresis

The two types of electrophoresis are moving-boundary and zone electrophoresis.

As discussed later, electrophoresis requires an enclosed chamber, support medium, buffers, power source, and a method for quantifying the amount of dye (e.g., elution method or densitometry). The two types of electrophoresis are *moving-boundary* and *zone* electrophoresis. Moving-boundary electrophoresis is rarely used because its primary uses, quantitative determination of protein mobilities and isoelectric points, have limited practical applications. Emphasis in this chapter will be placed on zone electrophoresis. There are two major types of zone electrophoresis: *paper electrophoresis* and *gel electrophoresis*. The first will be covered briefly, with major emphasis focused on gel electrophoresis.

Paper Electrophoresis

Although this method is seldom used today, it can serve as a simple illustration of the basic principle of electrophoresis. A piece of paper (cellulose) is dipped in buffer solution and then placed in a specially made tank to prevent evaporation (see Figure 1-2). The paper, available commercially, should have high wet strength and be of intermediate thickness. A protein sample is applied with a pipet as a spot or a line to one end of the paper, then low voltage (e.g., 20 V/cm) is applied. After separation of substances in the sample, the paper is removed from the tank and air-dried. Each component of the sample is identified by its color or fluorescence, or by staining with various dyes. A radio-labeled sample may be quantified by cutting up the spots and counting radioactivity.

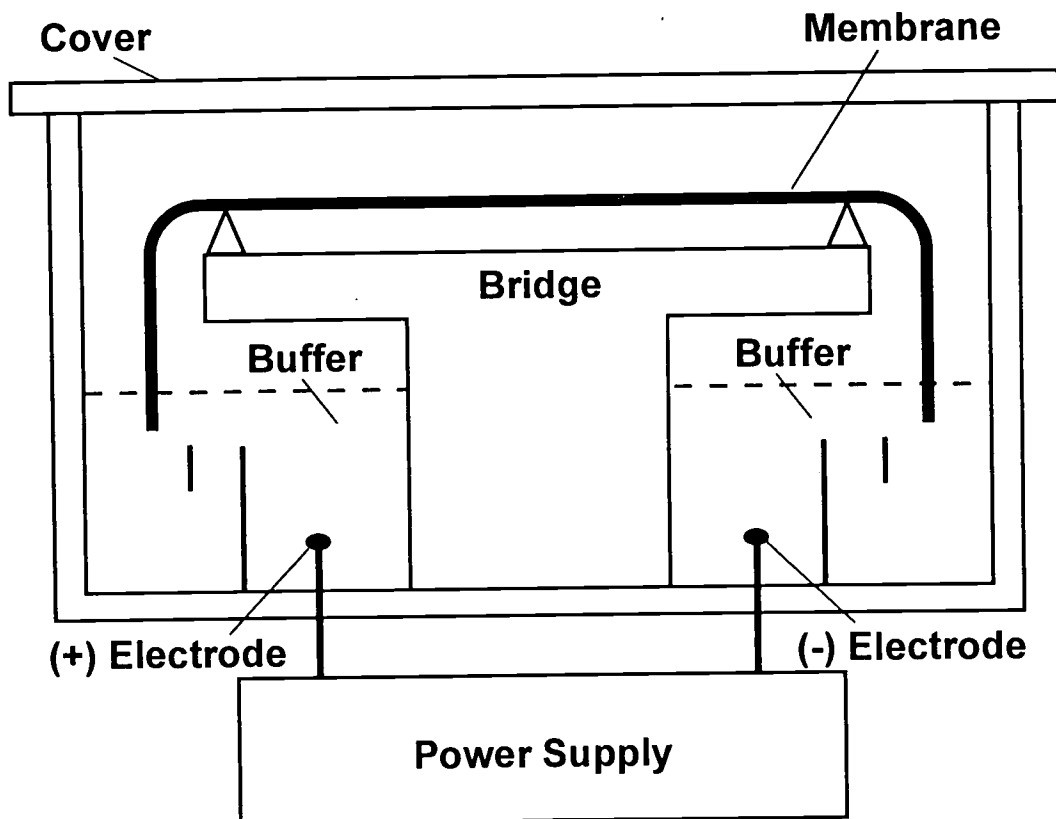


Figure 1-2
Diagram of an electrophoresis chamber.

6 Electrophoresis

Autoradiography of the entire sheet may also be carried out using X-ray film. Seldom used today, the primary use of low-voltage paper electrophoresis was analysis of protein mixtures that were not easily separated by chromatography.

Adsorption of the sample to hydroxyl groups in paper compromises resolution.

As protein separation evolved, another type of paper electrophoresis developed: cellulose acetate strip electrophoresis. This method employs conversion of hydroxyl groups in paper to acetate groups. Because protein samples often adsorb to hydroxyl groups, their movement is impeded, thereby allowing separation of different proteins. However, smearing of spots or bands, called tailing, can occur, which compromises resolution. Acetate groups are generally non-adsorbing so resolution is greatly improved. Sample spots are small and therefore more concentrated. Since cellulose acetate can be dissolved in various solvents, elution of material is a simple matter. Despite the advantages of using cellulose acetate over cellulose alone for electrophoresis, maximum resolution of protein samples can be obtained only through use of gel electrophoresis.

Gel Electrophoresis

Molecular sieving is separation based on molecular size.

Since resolution, or sharp separation of a sample into its component parts, is the objective of electrophoresis, more advanced methods have evolved over time. Use of gels has become the most widely used method for isolating proteins and nucleic acids because diffusion of the sample into the supporting medium is reduced and because gels separate substances based on the principle of molecular sieving, which is based on molecular size.

Electrophoresis of proteins was originally carried out on *starch gels*, consisting of a paste of potato starch whose grains had been ruptured by heating in a buffer solution. This medium consists of a wide range of pore sizes and consistency, and its opaqueness compromises quantification of separated proteins or enzymes. This technique provided the first evidence for the existence of isoenzymes. Today, for general protein separation, starch gels have been largely replaced by polyacrylamide gels.

Molecular sieving can be controlled by the concentration

Polyacrylamide gel electrophoresis (PAGE) has replaced starch gels for separation of proteins because molecular sieving can be controlled by the concentration of a polyacrylamide gel, and as mentioned previously, adsorption of proteins is negligible on a gel. Polyacrylamide is currently an effective supporting medium for separation of proteins, small RNA molecules,

PAGE is the acronym for polyacrylamide gel electrophoresis.

Variations of PAGE allow analysis and separation of proteins and RNA.

SDS-PAGE involves denaturation of proteins and breakage of disulfide bonds.

Proteins treated by SDS-PAGE behave as though they have uniform shape and charge-to-mass ratio.

and small DNA fragments. The medium is prepared by cross-linking acrylamide with *N,N'* methylenebisacrylamide in the container in which the electrophoresis will be carried out. Unlike the beaded form used in column chromatography, polyacrylamide is a continuous gel.

PAGE, using a column or a slab arrangement, can be carried out either horizontally or vertically. If gel concentration is low, the horizontal arrangement is essential, because a soft gel can actually be crushed under its own weight if in the vertical configuration. Slab gels have largely replaced column gels because this system allows a large number of samples to be separated simultaneously. Two variations of PAGE have been developed to affect analysis and separation of proteins and RNA molecules. These techniques are *sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)* of proteins and *polyacrylamide or polyacrylamide-agarose gel electrophoresis* of single-stranded nucleic acids.

SDS-PAGE involves denaturation of proteins and breakage of disulfide linkages to form a random-coil configuration. This technique, first described in the scientific literature in 1967, is based on the principle that molecular weights of most proteins can be determined by measuring their mobility in polyacrylamide gels containing a detergent, sodium dodecyl sulfate (SDS). The technique was improved by Weber and Osborn in 1969 (Weber & Osborn, 1969) and has since become a standard method for determination of molecular weights of single polypeptide chains. At neutral pH, in 1% SDS and 0.1 M mercaptoethanol, many multichain proteins will bind SDS and dissociate. Mercaptoethanol breaks disulfide linkages, resulting in loss of secondary structure. The remaining complex, consisting of protein subunits and SDS, assume a random-coil configuration (Figure 1-3).

The key concept is proteins that have been treated in this way behave as though they have uniform shape and identical charge-to-mass ratio. The reason is that each unit weight of protein binds with a constant amount of SDS (1.4 g SDS/g of protein). The charge on the protein is then determined by the bound SDS rather than the intrinsic charge on each amino acid. Since SDS-bound random coils all have the same charge-to-mass ratio, one might expect the electrophoretic mobility to be proportional to the molecular weight of the protein. However, the gel is a molecular sieve, and each molecule of protein must pass through it. Smaller molecules find their way more easily through the

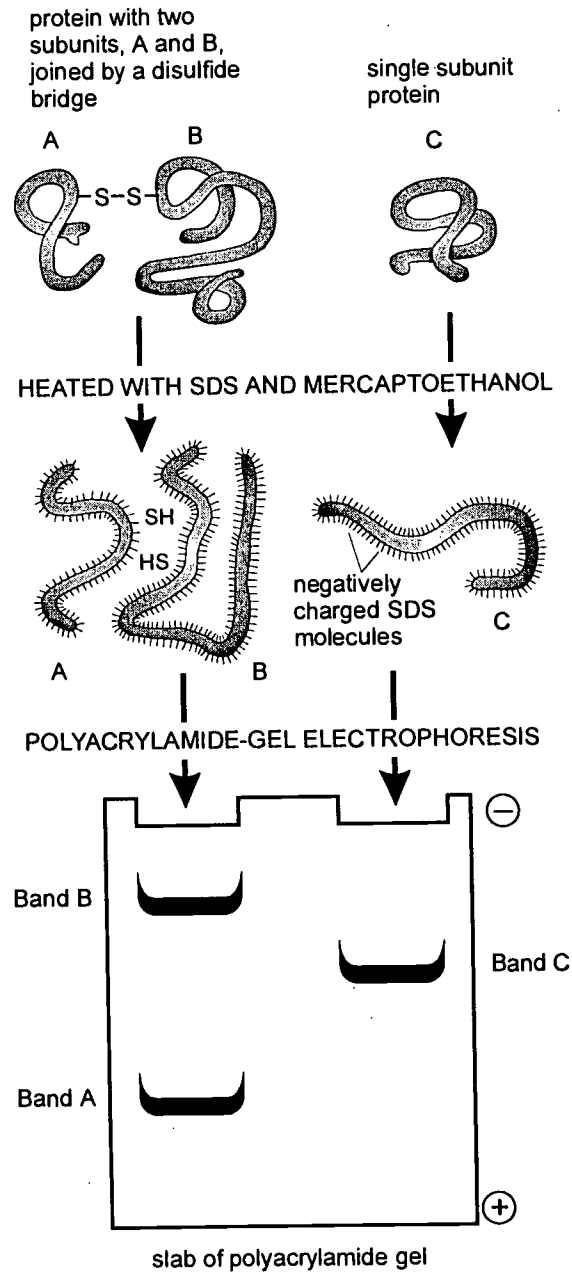


Figure 1-3

The principle of SDS polyacrylamide gel electrophoresis. Protein subunits bind with a constant amount of SDS, and mercaptoethanol reduces disulfide bonds, causing the subunits to dissociate.

gel labyrinth; thus, mobility *increases* as molecular weight *decreases*.

It has been shown that if a series of proteins of known molecular weights is electrophoresed in a gel, it will separate into a series of bands. A plot of the distance migrated versus the logarithm of the molecular weight yields a straight line. Therefore, if a protein of *unknown* molecular weight is electrophoresed with two or more proteins of *known* molecular weight (standards), the unknown can be calculated, usually to an accuracy within 5-10%. Accuracy increases if the number of standards is increased, and especially if two of the known molecular weights bracket the protein of interest. SDS-PAGE is the most common technique in use today for estimating molecular weights of proteins. The banding pattern typically seen in protein electrophoresis is shown in Figure 1-4.

SDS-PAGE is most commonly used to estimate protein molecular weights.

Polyacrylamide or polyacrylamide-agarose gel electrophoresis of single-stranded nucleic acids (RNA and some types of DNA) is based on the principle that each phosphate group carries a strong negative charge that far outweighs any charges on the bases. Therefore, charge-to-mass ratio of all polynucleotides is independent of base composition, and the molecular sieving effect of the gel is a major factor in separation of nucleic acids. Thus, smaller molecules will move faster than larger ones.

Separation of nucleic acids is based on the principle that each phosphate group carries a single negative charge.

Naturally occurring nucleic acid molecules are usually quite large, so gel concentration must be very dilute to form large pores in the medium. To strengthen the gel, agarose (a highly porous polysaccharide) is added, or sometimes agarose is used alone. Electrophoresis is done in slab or column gels. The distance migrated by the fragments is related to their molecular weights, and determination is made by including two samples of known molecular weight. Radio-labeled nucleic acid bands may be detected in gels in a number of ways. The gel may be sliced and solubilized in sodium hydroxide or hydrogen peroxide, added to an appropriate scintillation cocktail, and counted with a scintillation counter. If sample material is of high molecular weight, the gel concentration will be adjusted to make it highly porous, enabling molecules of protein to pass through it. Porosity will cause the gel to be soft; therefore, in such a case, the gel may be dried and firmly pressed against autoradiographic film and viewed.

Agarose, a polysaccharide, forms large pores when added to polyacrylamide gel.

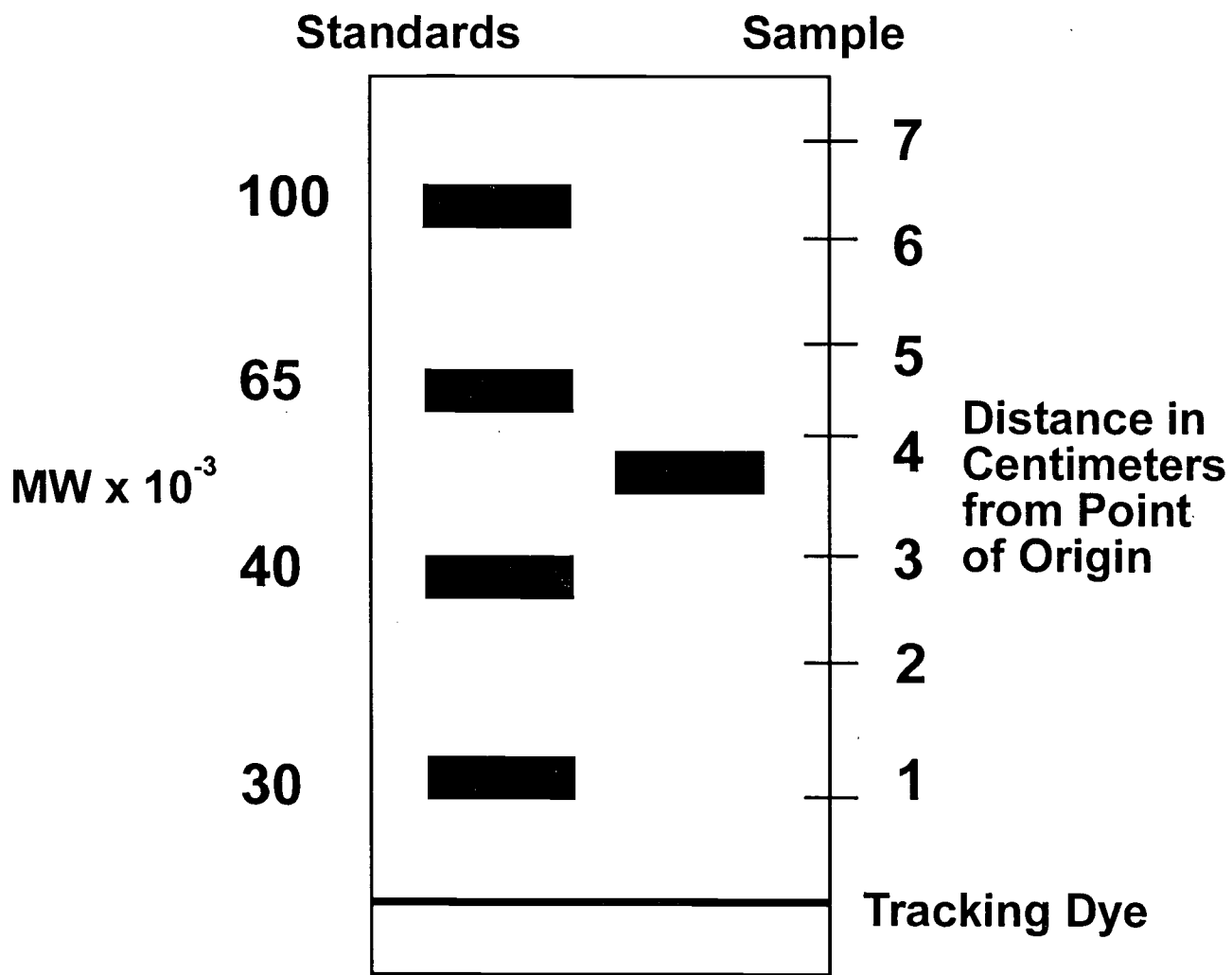


Figure 1-4

Estimation of molecular weight of a protein using SDS-PAGE. Black rectangles represent standard and simple protein bands.

Agarose-Gel Electrophoresis of Double-Stranded DNA

Agarose gel electrophoresis of DNA is instrumental in exploration of the genetic code.

A dalton is an atomic mass unit.

DNA from a human cell has over one billion base pairs.

Apoptosis, or programmed cell death, is the mechanism of normal cell turnover.

DNA technology has moved to the forefront of science and newspaper headlines. The introduction of agarose gel electrophoresis of DNA in 1970 has been instrumental in exploration of the complexities of the genetic code. Agarose gels, with their high porosity, allow penetration of DNA molecules with molecular weights up to 150×10^6 daltons. DNA fragments can be separated according to molecular weight or shape. Agarose gel electrophoresis may also be used for identifying the base pair sequence of large fragments of DNA. Electrophoresis may be employed to localize a specific gene in a fragment of DNA via a transfer-and-hybridization technique called the *Southern transfer*, named for Edwin Southern, who developed the technique. Similar hybridization techniques were subsequently developed for the study of RNA and proteins and were humorously termed *Northern and Western transfers*, respectively.

How would DNA fragments for gel electrophoresis be obtained, and why separate them from one another? A common source is the product of bacterial restriction endonucleases. Bacteria produce enzymes that recognize specific DNA sequences. Since an endonuclease always cuts the DNA at or near its unique recognition sequence, fragments of exactly the same size and number will result from a given sample of DNA (Figure 1-5). A small bacterial plasmid consisting of a few thousand base pairs will yield a few DNA fragments. DNA from a human cell has over one billion base pairs and will yield a million or more fragments. Agarose gel electrophoresis enables the investigator to separate fragments so that their size and number can be determined. Subsequent testing can then be carried out to determine the site of specific genes or transcription factors.

Another application of DNA electrophoresis is determination of the nature of DNA fragmentation that occurs when cells die. Normal cell death is termed "programmed cell death" or *apoptosis*, and DNA from these apoptotic cells exhibits a distinct banding pattern. This pattern is caused by cleavage of DNA into oligonucleosome-length fragments by endonucleases found within the nuclei of cells and is similar to the bands produced by bacterial restriction endonucleases. When cell death is due to toxicity or injury, it is termed *necrosis*, and DNA from these cells appears smeared rather than resolved into distinct bands when electrophoresed in an agarose gel. Thus gel electrophoresis of DNA is a valuable tool for determining the mechanism of cell death in normal or pathophysiological states.

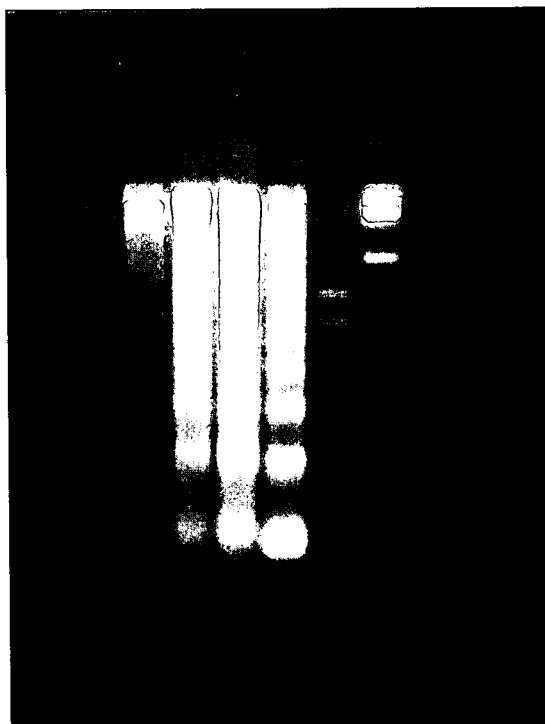


Figure 1-5

Endonucleases cut DNA, resulting in the same size and number of fragments from a given sample of DNA. Photograph of fragmentation banding pattern seen in apoptotic DNA stained with ethidium bromide when viewed under UV light.

Agarose gel concentration directly influences pore size and, thus, DNA migration patterns.

As mentioned previously, the phosphate backbone of DNA causes the molecule to carry a net negative charge. When voltage is applied across an agarose gel into which DNA samples have been loaded, they will migrate toward the positive electrode (the anode). The frictional force imposed by the gel matrix impedes migration of the fragments. Since the charge-to-mass ratio is identical for all DNA molecules, it is their length that determines how fast they will move. Long fragments will migrate more slowly than short ones. The concentration of the agarose gel, which directly influences its pore size, is also another important factor influencing DNA migration. For large fragments of about 2,000 to 20,000 base pairs, the gel concentration should be about 0.6% (0.6 g agarose/100 ml buffer). If fragments are smaller (200-4,000 base pairs), then a concentration of 1.5% is used. In general, for most DNA separations, the gel concentration ranges from 0.8 to 1.0%.

DNA migrates at a rate proportional to the applied voltage.

Although DNA will migrate at a rate that is proportional to the voltage applied to the medium, it must be remembered that as voltage increases, larger fragments will migrate at a rate proportionally faster than smaller ones. Thus, at high voltage, large DNA fragments are not as well resolved (separated) as smaller ones. Gel temperature will also influence migration. The higher the temperature, the faster the migration. Since an increase in voltage causes heat production, it is best to run the gel at no more than 7 V/cm. A typical voltage is 1.0 to 1.5 V/cm for about 16 hours; though small fragments, such as those from apoptotic cells, can be well resolved in only two hours at 7 V/cm.

Most DNA electrophoresis is performed using horizontal gel slabs. Gel tanks usually consist of two rectangular buffer tanks made from acrylic plastic separated by a horizontal plastic plate that extends from one buffer tank to the other (see Figure 1-6). On one end of the tank apparatus, the positively charged anode is mounted and, on the other end, is the negatively charged cathode. The gel is made by adding electrophoresis-grade agarose powder to an electrophoresis buffer solution. This mixture is heated until a uniform, molten slurry is formed. Ethidium bromide, a dye that permits visualization of separated DNA bands, may be added to the gel at this time or after electrophoresis is finished. The agarose mixture is then poured into a prepared mold that has a plastic comb near one end with teeth held 0.5-1.0 mm above the bottom of the mold that will form wells to hold DNA samples.

Basic Electrophoresis Apparatus

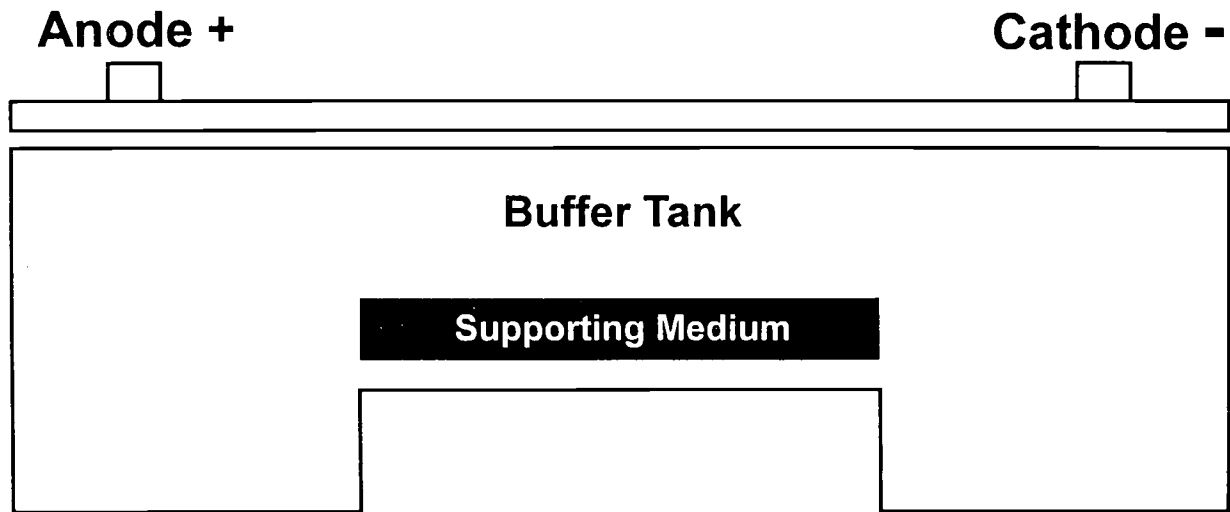


Figure 1-6

Typical buffer tank apparatus for gel electrophoresis of DNA. The part of the diagram labeled "supporting medium" represents the gel on which samples are loaded.

Ethidium bromide is a dye that permits visualization of separated DNA bands.

A typical gel is 10-20 cm long and 0.5-1.0 cm thick. After allowing the agarose to “set” for approximately one hour, the comb is carefully removed, and the plate containing the hardened gel is placed on the plastic extension between the two buffer tanks. Commonly used running buffers are Tris-acetate or Tris-borate with EDTA, a metal chelator. Buffer is poured into the tanks until it covers the gel to a depth of several millimeters. This type of setup is called a “submarine” or *submerged gel*. The anode buffer tank will become alkaline during electrophoresis. Therefore, it is advisable to recirculate buffer between the two tanks with tubing connecting the two buffer tanks.

A running gel apparatus should remain covered and not be moved during operation.

Before DNA samples are placed into the wells of the gel, they are mixed with loading buffer that typically consists of bromophenol blue and glycerol in water. The DNA mixture is carefully pipetted into the wells under the buffer, a skill that requires some practice. The glycerol in the DNA solution increases its density, causing it to fall into the well, and bromophenol blue allows for easy visualization of the DNA solution during loading. After loading, the plastic cover is placed on the buffer tank apparatus, the gel apparatus is placed away from work areas, and the voltage is adjusted as previously described. To prevent electric shocks, the running gel apparatus should remain covered and should not be moved during operation.

Ethidium bromide binds DNA by intercalation, or slipping between stacked bases.

When electrophoresis is finished, the power supply is turned off and electrodes detached. The gel may be carefully slipped from the plate on which it is mounted into an appropriately sized dish filled with buffer. As mentioned before, ethidium bromide permits visualization of the DNA bands. It binds to DNA by slipping between the stacked bases, a process termed *intercalation*. The bands then fluoresce brightly when exposed to ultraviolet light (wavelength 260-360 nm). Ethidium bromide is toxic because of its ability to bind to DNA tightly. It is therefore a mutagen and can be hazardous to laboratory personnel. **Gloves should always be worn when handling gels or solutions containing ethidium bromide.** Also, since ultraviolet (UV) light can cause skin cancer and blindness from damage to the retina, protective eyewear should be worn and exposure to UV light should be kept to a minimum. A photograph of the gel during exposure to UV light is usually taken for purposes of identification and quantification. A photograph of a typical agarose gel showing the characteristic banding is shown in Figure 1-5.

Protective eyewear should be worn when viewing gels exposed to UV light.

A mixture of DNA standards is called a ladder.

To quantify the size of DNA fragments separated on an agarose gel, standards of known size are typically run in one or two of the lanes along with the DNA sample. A useful standard is a mixture of DNA fragment sizes called a *ladder*. Molecular weight of each fragment is determined by measuring the distance migrated from the origin. The size of each fragment in kilobase (kb) pairs is known from comparison to standards. The \log_{10} of the molecular length (in kb) of each DNA fragment is graphed against the distance migrated in the gel. When the points are connected, those in the middle of the graph usually form a straight line, and the predicted molecular weight may be read from the Y-axis.

Agarose gel electrophoresis of DNA is simple in theory, but it is one of the more important molecular biology techniques in use today. This technology represents a quantum leap in our ability to gain a deeper understanding of our genetic code.

Questions Commonly Asked by Students

Question 1: Why is it necessary to separate proteins and nucleic acids from one another?

Proteins and nucleic acids are separated from one another for identification, purification, and for determination of molecular weight and mechanisms of disease associated with changes in proteins or DNA.

Question 2: Why does electricity cause proteins and nucleic acids to move?

Electric current causes proteins and nucleic acids to move because these substances carry a charge and are suspended in a medium between a positive and negative field. When an electric field is applied across the medium, the substance will be drawn by electrostatic attraction to the opposite charged pole.

Question 3: Why is acrylamide or agarose gel the preferred supporting medium in electrophoresis?

Gels are used for electrophoretic separation because their pore size can be controlled by the concentration of the gel, and there is virtually no adsorption of sample to a gel, allowing for better resolution.



Terry S. LeGrand, Ph.D. candidate (left), and Dr. Tak Yee Aw (right) demonstrate electrophoresis to Gina Vallient, graduate student in the Department of Physiology and Biophysics at the LSU School of Medicine in Shreveport, LA.

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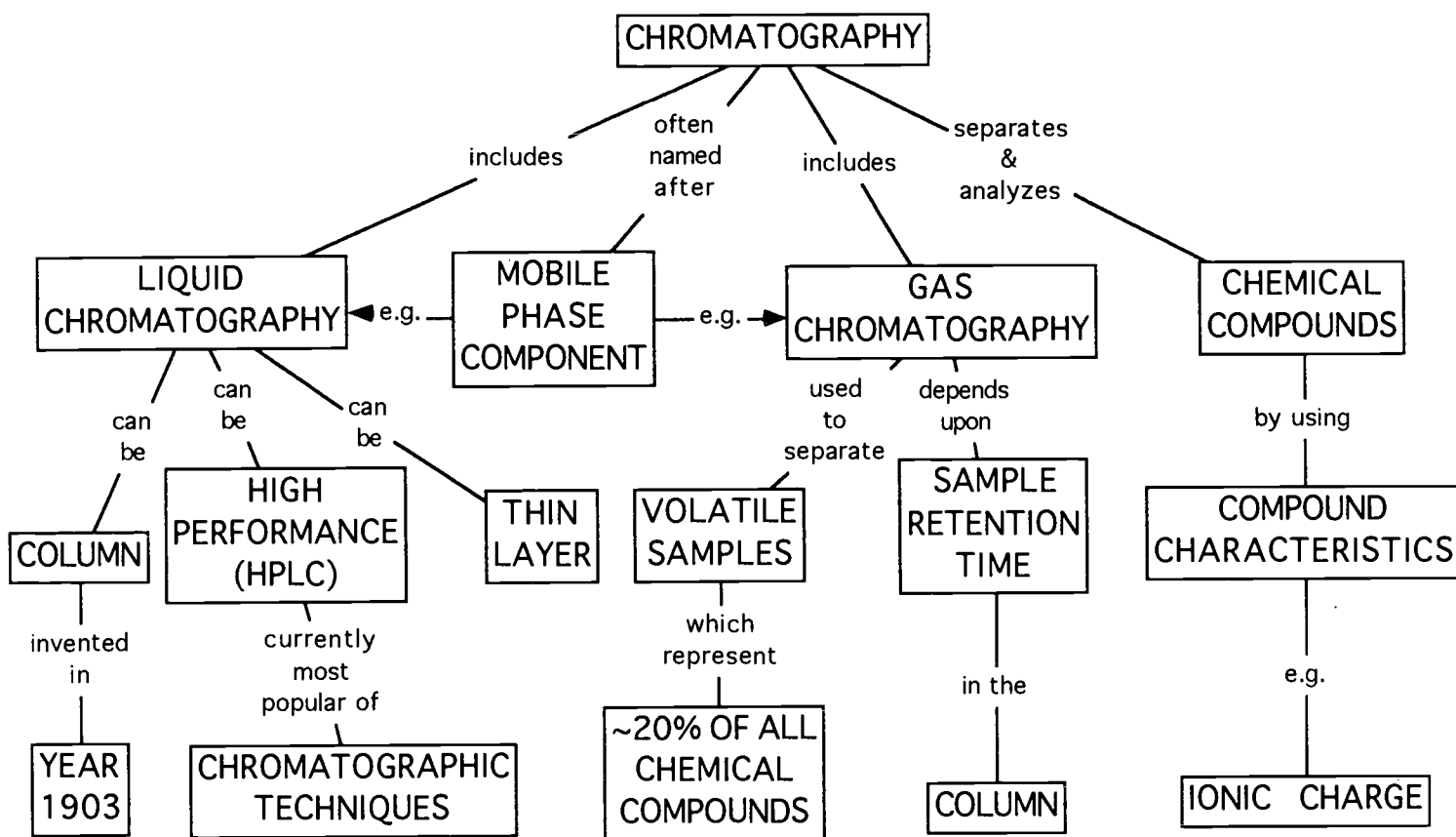
CHROMATOGRAPHIC TECHNIQUES

2

Suzanne Childers Huth

Overview

1. Origins of chromatography
2. Liquid chromatography
3. Gas chromatography



Chromatographic Techniques

Chromatography is an analytic method used to separate and identify the components of a chemical mixture.

Chromatography may be defined as a method used to separate compounds. The basis of this separation is the characteristic differences of various compounds as they travel through a particular type of supporting medium. These differences are revealed as the compounds react, within the supporting medium, with predetermined mobile phase and stationary phase materials. The mobile phase material may be either liquid or gaseous in composition, and the stationary phase may be a solid, a liquid, or a liquid coated on to a solid. The support medium most commonly used is a solid substance (see Figure 2-1).

The beginning of chromatography has been traced to the work of a Russian botanist by the name of M.S. Tswett in 1903. The first documented account of his work illustrated the use of a glass cylinder or column that had been packed with a fine powder. An extract of chlorophyll made from plant leaves was placed on the top of the column, and a liquid consisting of heptane and ethanol was poured, at a slow, steady pace, through the column. By use of this technique, four different chlorophyll pigments were separated into colored bands of gray and green hues. These "colored" bands were responsible for the derivation of the name *chromatography*.

Since its humble beginnings, the process of chromatography has had numerous refinements and variations. Many different techniques have been developed from this early work. Often, the specific type of technique to be used is defined by the type of mobile phase material to be used. For example, if gas is used as the mobile phase component, then the technique is referred to as *gas chromatography*. If, on the other hand, the mobile phase material is liquid, then the technique is termed *liquid chromatography*. Other techniques may be based on the type of support medium used, the type of solid material used, or the added refinements of automated technologies. Liquid chromatography and gas chromatography are the two most commonly implemented types of fundamental techniques. These techniques are the focus of this chapter.

Liquid Chromatography

Liquid chromatography uses a liquid mobile phase to separate compounds.

Liquid chromatography, or LC, as it is often referred to, is characterized by the use of a liquid mobile phase to separate the sample compounds. The instrumentation required for these

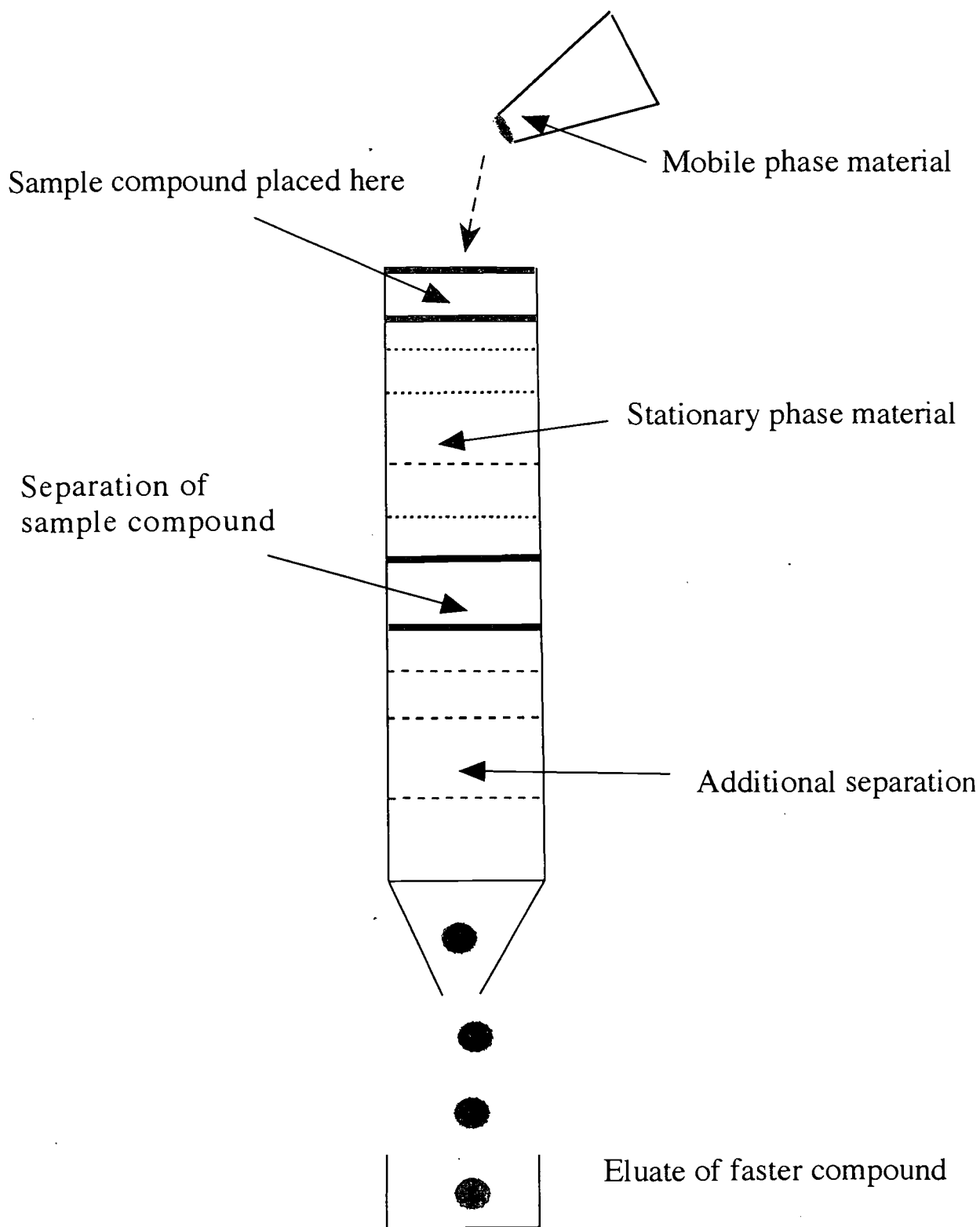


Figure 2-1
Separation of sample compounds by chromatography.

techniques includes an injector, a column, a pump, a mobile phase material, a detector, and a recorder. The method of injection can be as simple as pouring the sample into the top of the column, as is demonstrated by an open system such as the one used by Tswett. If a closed system is involved, the sample may be injected by the use of a syringe into a septum or port.

The columns can be made of many types of materials. They may be made of metal, glass, or plastic. The shapes may also vary from straight to coiled. The columns are usually packed with a supporting medium which will allow for the best separation possible. This medium is selected on the basis of the type of compounds that are to be separated. The supporting media most commonly used consist of inert materials such as glass or powder. The column must be constructed in a way that allows for the implementation of a proper liquid mobile phase as well as a proper stationary phase. These, as well, are selected on the basis of the various compounds to be analyzed.

Columns can be made of many types of materials such as metal, glass, or plastic.

A pump component is used to deliver the mobile phase into the unit at a constant rate. It may be delivered via gravity (as implemented in low pressure techniques) or some type of mechanical pump. Mechanical pumps are used in high pressure techniques. These may be either a syringe which is governed by a regulator pump or a type of reciprocating pump that uses a cyclic approach to mobile phase delivery.

The mobile phase material will be termed as being either a weak or strong solvent. If the mobile phase material is defined as being strong, then the solvent will elute the compound from the column very rapidly. If, on the other hand, the mobile phase material is defined as being weak, the elution process will be slow. Thus the weaker the mobile phase material, the slower the elution process through the column and the longer retention rate within the column.

To describe how solutes are retained on a column with different solvents, the terms weak mobile phase and strong mobile phase are used.

The detector systems may be of several different types. Detectors may be thermal conductivity detectors, flame ionization detectors, argon ionization detectors, fluorescence detectors, or electron capture detectors. The selection of the type of detector to be used depends on the type of substance to be measured. Detector selection will be discussed in more detail later in this chapter.

The purpose of the detector is to help identify the unknown compounds.

Dedicated computer systems
make the best recording
systems for LC.

The recording system can consist of a strip-chart recorder, an integrator, or a dedicated computer. As one might imagine, the most versatile, as well as the most popular, type of recording system is the dedicated computer. The flexibility of the computer program allows both simple and complex data to be represented on the chromatographic profile.

With the use of these components, a sample can be separated based on its interaction with the environment of the column, the interaction can be interpreted by the detector, and all of the data accumulated can be plotted in graphic form by the recording system.

Of course, the real key to the usefulness of the procedure is the degree of separation accomplished in the column. There are five basic techniques used for sample separation in liquid chromatographic methods: adsorption (liquid-solid), partition (liquid-liquid), ion-exchange, size exclusion (stearic), and affinity binding.

Techniques for Separation Used in Liquid Chromatography

Adsorption

The method of adsorption separation requires a liquid mobile phase material and a solid stationary phase material. The stationary material adsorbs solutes but lends itself to a reversible process. This same material is used as the support medium. An example of this type of medium is silica. Although not used to a great extent, this type of separation does have the advantage of being able to separate material which cannot be separated by other techniques. It is particularly useful when the components being separated vary extensively in polarity.

Partition

A method which makes the use of a liquid mobile phase and a liquid stationary phase with a solid support medium is referred to as liquid-liquid separation or *partition chromatography*. This was one of the first techniques to be commonly used by scientists and is still used in many labs today. The principle of separation in this technique is based on the concept of polarity. A highly polar substance tends to be more soluble in a highly polar liquid such as water.

Partition or liquid-liquid
chromatography separates
compounds based on their
partitioning between a liquid
mobile phase and a liquid
stationary phase.

Partition chromatography can help analyze biological compounds such as amino acids and hormones.

A less polar substance tends to be more soluble in a less polar substance, such as the organic solvent benzene. Because polarity is so important, the H^+ concentration must be controlled. Thus, the pH must be regulated to maintain proper polar composition. The liquid stationary phase can be a polar liquid that can readily retain polar compounds or a nonpolar liquid that can retain both polar and nonpolar compounds. These techniques may be used quite easily to analyze biological components such as hormones, amino acids, and other small organic compounds (see Figure 2-2).

Ion-Exchange Chromatography

Ion-exchange chromatography is a liquid technique in which compounds are separated based on their charge and adsorption onto a support that contains a fixed charge.

Ion-exchange chromatography is a liquid chromatographic technique whereby compounds are separated on the bases of their ionic charge. In this technique, the stationary phase consists of a resin made up of large polymers of substituted benzenes, silicates, or cellulose derivatives. Cation-exchange resins or anion-exchange resins may be used. Both organic and inorganic materials may be analyzed using this technique. It is particularly beneficial in separating amino acids, isoenzymes, and miscellaneous organic ions. If both cation-exchange resins and anion-exchange resins are used, the deionization of water may be accomplished. In fact, the natural purification of water as it flows through soil is one of the best examples of this technique.

Size Exclusion Chromatography

Size exclusion chromatography separates molecules according to different sizes and shapes.

Size exclusion chromatography, or steric chromatography, is a technique used to separate molecules according to differences in their size and shape. The support medium used in this technique has a defined range of pore sizes. As the solutes travel through this support, molecules that are smaller than the pores will diffuse into the stationary material, while those that are larger than the pores will flow through the column. Thus, the larger particles will be the first to elute. A common use for this type of separation is that of separating proteins from peptides. It has also been used extensively in the separation of isoenzymes and enzymes.

Affinity Binding

The final type of separation technique used in LC is a specific type of adsorptive technique known as affinity binding. This is a liquid chromatographic technique which is based on the specific reversible interactions that occur between the binding of an immobilized molecule on a solid support medium to-

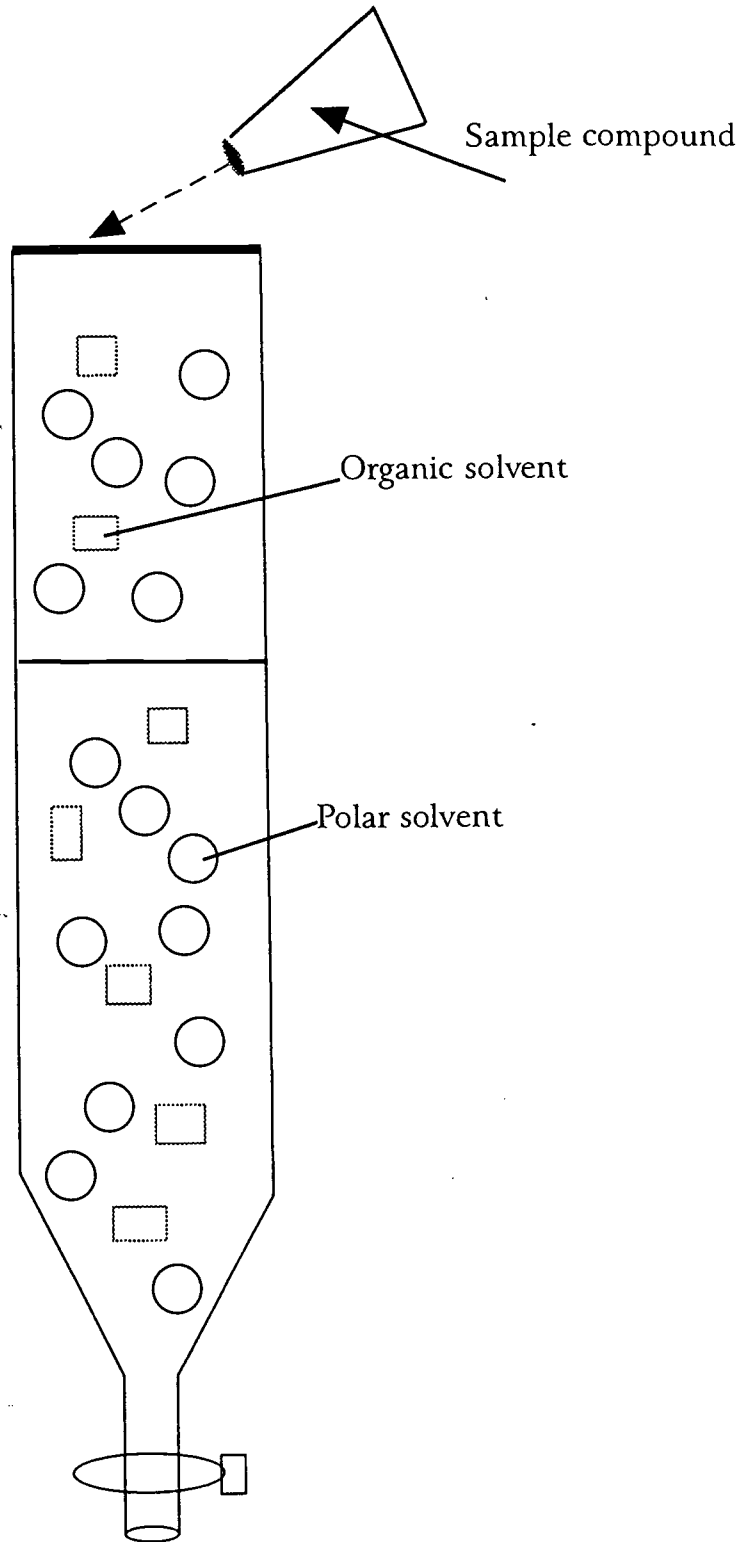


Figure 2-2
Partition chromatography.

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Affinity chromatography separates reversible interactions that occur between binding of enzymes with substrates or the binding of an antibody with an antigen.

ward the constituent to be separated. This immobilized molecule, known as the *affinity ligand*, is used as a selective adsorbent for the affinity molecule. This is the stationary phase, and it determines the specificity of the column. Ligands of high specificity, such as specific antibodies, antigens, and single-stranded nucleic acids, are useful for determination of corresponding antigens, antibodies, or specific proteins. One very interesting application of this technique is in the determination of glycosylated hemoglobin for the long-term management of diabetes.

The Most Commonly Used Types of Liquid Chromatographic Techniques

Column Chromatography

The first type of chromatography was liquid column chromatography.

Since Tswett used a liquid mobile phase in his plant extract experiment, the first type of chromatographic technique ever used was liquid chromatography, or as first recorded, column chromatography. In the example depicted from Tswett's experiment, the powder packed in the glass cylinder acts as the stationary phase while the liquid material of heptane and ethanol (the solvent) acts as the mobile phase. As the bands are separated through the column, the solute (consisting of separated materials) is removed from the stationary phase. This removal of the solute from the stationary phase is a process known as *elution*. The mobile phase is referred to as an *eluant*, while the solute coming through the column is the *eluate*.

Identification of the sample being analyzed is determined from a chromatograph.

As sample components travel through the column, each will interact differently with the mobile phase and the stationary phase. If the component reacts stronger with the stationary phase, it will stay in the column longer than a component that reacts stronger with the mobile phase. Thus, separation is a function of both retention time and void time. These values that correspond to the characteristics of a compound are directly related to the strength with which it interacts with the stationary and mobile phases. When these values are measured by the use of a detecting mechanism, the values may be plotted on a representative graph known as a *chromatograph*. Using these values, along with a measurement of peak area on the graph, the identity of a component may be determined.

As one can imagine, this type of procedure can be a long and arduous one. However, due to the unlimited possibilities

foreseen by various scientists along the way, the column chromatographic procedure was refined and improved to include a wide variety of procedures.

Thin-Layer Chromatography

Thin-layer chromatography uses a thin layer of gel as the stationary phase.

Thin-layer chromatography is a technique that uses a glass or plastic plate (often called a flat bed) coated with a thin layer of gel (see Figure 2-3). This thin layer of gel is known as the sorbent or the stationary phase. The gel may be composed of alumina, cellulose, dextran, or silica. The sample to be analyzed is applied as a spot near one edge of the plate. The solvent or mobile phase component is placed in a closed container so that the vapor from the solvent can permeate the space within the container. The plate is then placed in the container so that the edge on which the sample was applied is touching the solvent. The solvent migrates, by capillary action, up the plate, carrying the dissolved sample molecule with it. After migration is complete, the plate is removed and dried. The unknown sample or samples may then be compared with known samples and analyzed according to these standards. Some sample components may overlap. If this occurs a staining agent may be applied to aid in the identification of the components.

Thin-layer chromatography is used in drug testing and assessing fetal lung maturity.

Thin-layer chromatography is commonly used as a screening procedure for mass analysis of single samples or mixed samples. It is of particular importance in drug screening procedures and fetal lung maturity testing.

High Performance Liquid Chromatography

HPLC maximizes the results of LC.

One of the most popular chromatographic techniques today is high performance liquid chromatography (HPLC) (see Figure 2-4). A pump is used in this technique to move the mobile phase through the column at a much greater speed than a traditional gravity flow column. A fine, uniformly packed column is housed in an oven to control the temperature and enhance the rate of separation. A detector generates electrical signals representative of the concentration of the eluted compound. The data are collected by a recorder and then may be plotted as a chromatograph. HPLC's controlled environment lends itself easily to the processes of automation. Using this technique in an automated form has been extremely beneficial in medical laboratories to identify analytical components in mass numbers. Not

HPLC is used in drug monitoring and endocrine testing.

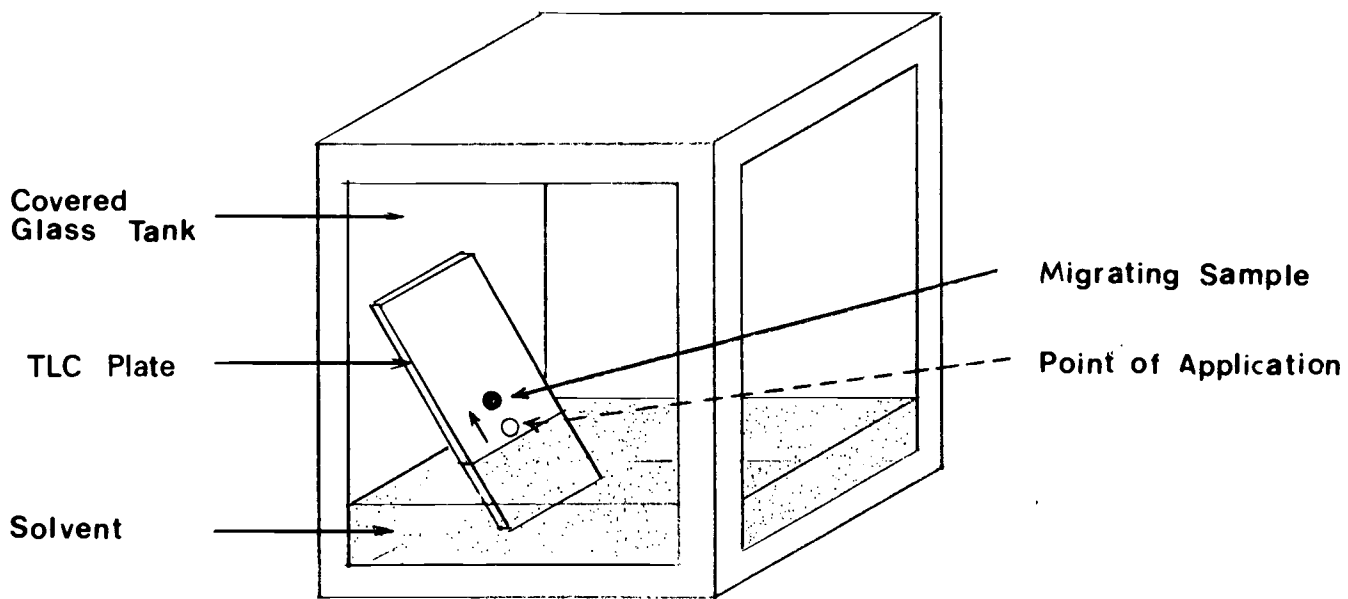


Figure 2-3
Thin-layer chromatography.

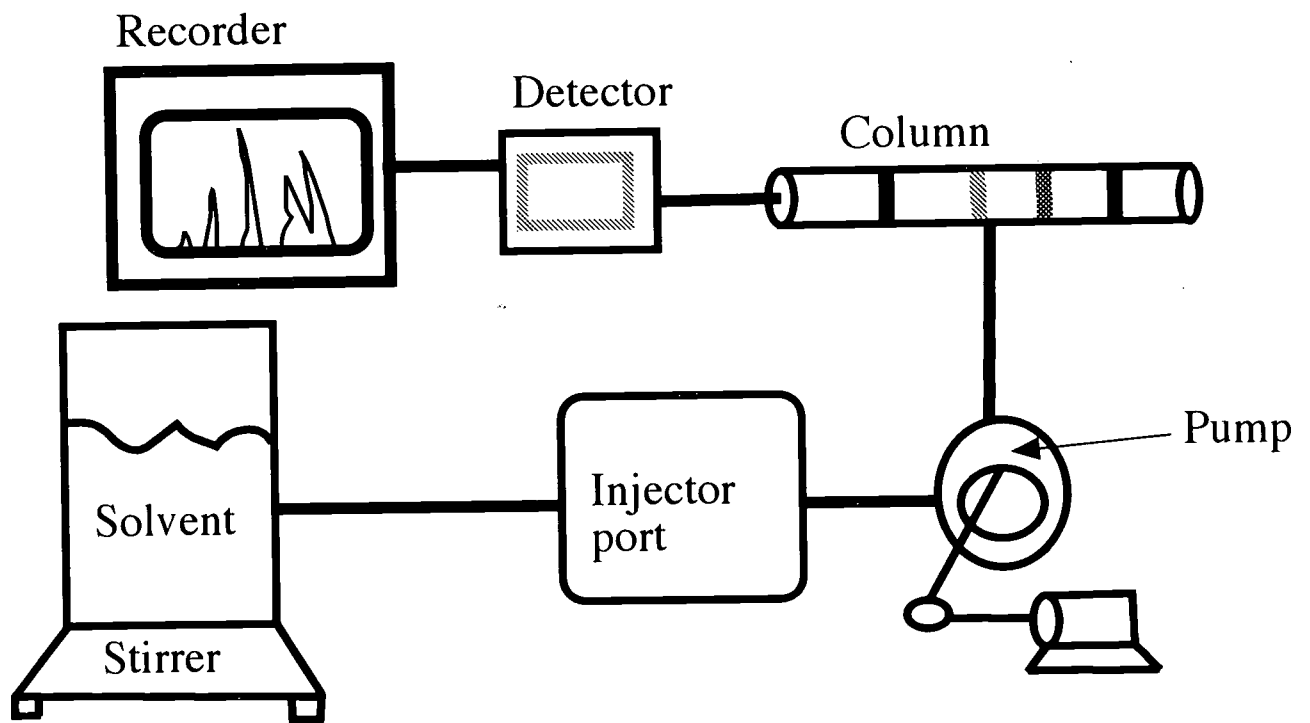


Figure 2-4
High performance liquid chromatography.

only is it a very sensitive and accurate procedure, but it is also significantly faster than most liquid chromatographic techniques.

Gas Chromatography

Volatile compounds can be separated by gas chromatography.

Gas chromatography is based on the gaseous state of the mobile phase of this technique. It is used to separate mixtures of compounds that are volatile or those that can be made volatile. The instrumentation required for these techniques consists of five basic components which include an injector, a column, a mobile phase material, a detector, and a recorder (see Figure 2-5).

The Injector

The injector system may use manual injection or automated injection. The sample is injected through a heated rubber septum. Its temperature is generally maintained at a temperature 30° C above the temperature of the column. As the sample is injected, it is made volatile by the use of a flash heater and is swept through the column by a carrier gas. Injection may also be direct, with no heating. This is suitable for samples that are unable to withstand the heat of the flash heater; however, then the carrier gas must be preheated.

The Column

The column, which can be a packed column or a capillary column, is enclosed in an oven. The oven provides an insulated environment which allows for effective temperature control as uniform temperature. A fan may be incorporated to enhance the temperature regulation.

The Mobile Phase

A mobile phase source is used, and it normally consists of a gas cylinder filled with a carrier gas of the highest purity. The gases most commonly used are helium, nitrogen, hydrogen, and argon. The cylinder is equipped with meters used to control the flow of the gas and thus control the ultimate gas pressure through the column.

The Detector

The three most commonly used detectors in gas chromatography include the thermal conductivity detector, flame ionization detector, and electron capture detector. Each of these

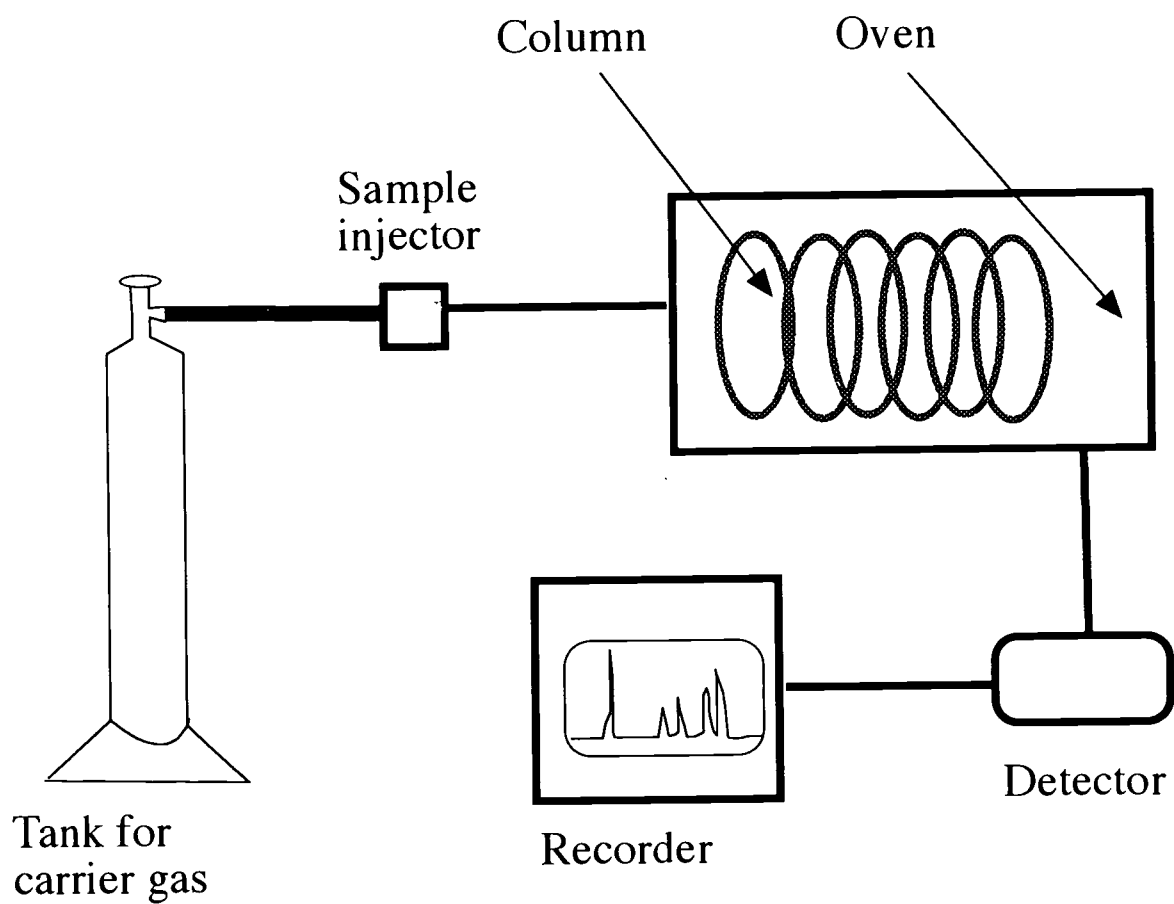


Figure 2-5
Gas chromatography.

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GC detectors measure physical, rather than chemical, properties.

mechanisms is based on the differentiating characteristics of the sample gas and the carrier gas, as each leaves the column. The thermal conductivity detector, for instance, is based on the knowledge that a heated sample will lose heat as the carrier gas is passed over it. By measuring the ability of the carrier gas to conduct heat away from the hot wire element, a change in the conduction can indicate a change in the compounds as they are eluted from the column. To aid in this detection, electronic circuitry is used to collect the data.

Flame ionization detectors are widely used to detect organic compounds.

The most common type of detector is the flame ionization detector. This detector identifies compounds by measuring their ability to produce various ions when burned in a hydrogen flame. The ions produced are gathered by an electrode that surrounds the flame. This creates a current that allows the eluting compound to be detected.

Electron capture detectors are used to detect electronegative elements, such as the halogens.

The electron capture detector is a radiation-based device for detecting compounds that contain electronegative elements such as halogen atoms. Radioactive sources which emit beta particles during their decay process are used. As the beta particles are released, they collide with the carrier gas. This allows the production of a high number of low energy electrons that can be collected and recorded until the sample is eluted from the column. As the sample elutes, electronegative particles from the sample begin to capture energy from the carrier gas electrons. This capture reduces the current and allows the sample gas to be detected.

The Recorder

The recording system is the last component of the gas chromatograph. It functions to process, record, and store the information collected by the detector. There are several types of recording system outputs. They may range from a simple graphic representation to a sophisticated computer analysis of the data. The type of detector used and the type of sample being measured play a significant role in the choice of recording systems.

The Process

The basic process of gas chromatography begins with the use of an inert substance (usually composed of hydrogen, nitrogen, helium, or argon) as the carrier gas. It carries the solute molecules through the column which is made up of a nonvola-

Retention time of the sample in the column is used in the identification of the sample.

tile liquid coated on an inert solid support medium. The sample to be analyzed is injected through a thermally controlled injection port sealed with a rubber septum. An independently heated metal block surrounds the injection port that allows heating of the sample to a significantly higher temperature than the column itself. The vaporized sample is then carried through the column partially as a gas and partially as a dissolved substance in the liquid phase. The more volatile the compound, the quicker it moves through the column. Compounds that are less volatile will spend more time in the stationary liquid phase and will move slower through the column. This degree of difference is referred to as the *retention time* and is used in the identification of the sample. The degree of volatility depends on the boiling point of the compound; therefore, the temperatures within the column must be tightly controlled. As the gas passes through the detector, an electrical signal, proportional to the amount and specific characteristics of the sample contained, is produced and recorded.

Advantages and Disadvantages

Only 20% of all known compounds are suitable for gas chromatographic analysis.

There are pros and cons to both liquid chromatography and gas chromatography. The biggest disadvantage of gas chromatography is that it is limited to separating compounds that can be converted to a volatile derivative. Under 20% of all known compounds fall into this category; therefore, liquid chromatography is the more commonly used method at the present time. Another disadvantage of gas chromatography is the lengthy amount of time needed for sample presentation and for construction of the column. The advantages of gas chromatography include speed, sensitivity of small sample size, and availability of ready automation. Advantages of liquid chromatography include a wide range of sampling size, the use of lower temperatures, and a great deal of flexibility in the changing of both mobile phase and solid phase materials. Disadvantages include poor sensitivity to certain compounds and the high expense of equipment needed to run the analysis.

Summary

The key to a successful analysis is selecting the proper type of chromatographic technique to be used. This depends on the characteristics of the component being measured and the quality of measurement which needs to be obtained. De-

degrees of measurement may range from qualitative to semi-quantitative to quantitative values. As has been shown, the uses of chromatographic separation techniques are broad in scope. They range from identification of characteristic amino acids to the quantitative analysis of drugs, hormones, steroids, and carbohydrates. The biological applications are virtually limitless.

Questions Commonly Asked by Students

Question 1: What if two compounds are so stable that they cannot be separated by chromatographic techniques?

Other techniques may be used, such as mass spectrophotometry or atomic absorption photometry.

Question 2: What can be done to increase the resolution of separation?

The resolution may be increased by using a longer column, such as a capillary type. Use of a stationary phase compound that would retain the sample compound for a longer period of time can also increase the resolution.

Question 3: With so many types of separation techniques from which to choose, how are choices made for sample separation?

Techniques are chosen on the basis of several factors. The characteristics of the sample compounds may dictate the use of a specific technique. Other factors that are considered are the amount of time available for the assay, and the cost effectiveness of the procedure.



Suzanne Huth instructs Dan Anderson, a graduate student in the Department of Clinical Laboratory Science and Bacteriology at Louisiana Tech University, in the technique of chromatography.

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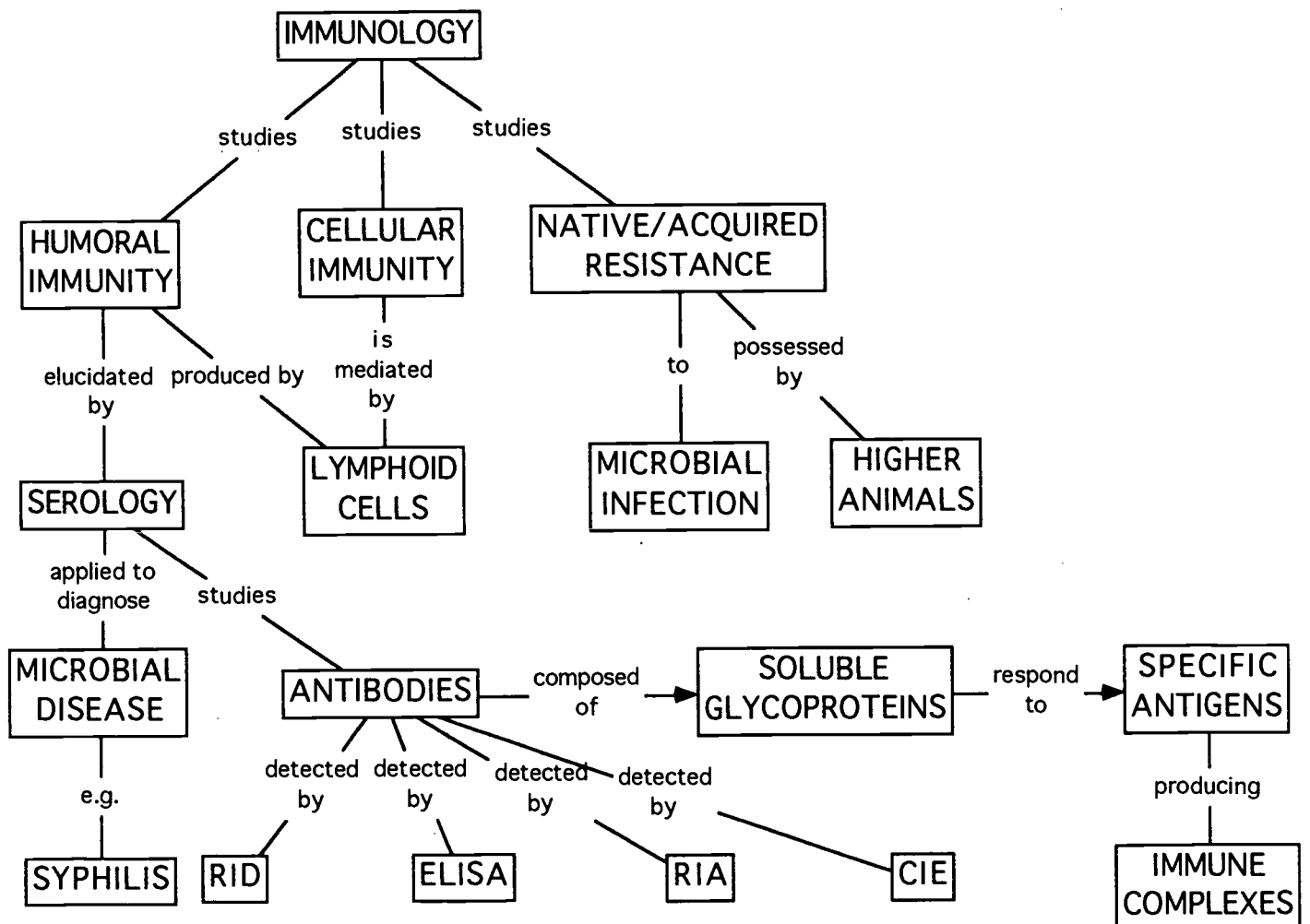
IMMUNOLOGIC METHODS

3

Selvestion Jimes

Overview

1. Immunologic assays
2. Immuno-electrophoresis
3. Agglutination
4. ELISA
5. Nephelometry
6. Countercurrent electrophoresis
7. Radioimmunoassay
8. Fluorescent antibody technique



Immunologic Methods

Introduction

The body has two distinct immune responses to an antigen: humoral and cellular.

The human body is exposed to a virtual sea of microorganisms beginning at the time of birth. In response to these microbes, the body has developed a unique defense mechanism against foreign substances — the immunologic response. This response is made up of two distinct parts: (1) humoral and (2) cellular. Humoral immunity is mediated by antibodies while cellular immunity is mediated by lymphoid cells or white blood cells. This chapter will focus on cellular immunity.

The study of immunity is called *immunology*; whereas “immunity” refers to an organism’s susceptibility to disease. Serology is a branch of immunology used for the detection and measurement of specific antibodies that develop in blood following exposure to a disease-causing antigen.

When an antigen is introduced into the body, it will result in the formation of a protein called an antibody.

Microorganisms can serve as antigens or immunogens. *Antigens* (Ag’s) are foreign substances that, when introduced into the body of an animal, will stimulate the formation of antibodies and/ or cytokines.

A hapten is a substance that cannot by itself stimulate antibody synthesis but can react with a hapten-specific antibody.

Haptens are substances that can react with antibodies but are unable to elicit an immune response. Small molecules can serve as “haptens” that have the ability to combine with protein in the skin. Once it combines with the protein carrier molecule, the complex becomes a complete antigen. Haptens play an important role in contact sensitivities to cosmetics associated with delayed type hypersensitivity due to production of cytokines by sensitized lymphoid cells (T cell lymphocytes). Cytokines are molecules that regulate the immune response by T cells, B cells, and macrophages.

Antibodies are found in plasma and are known as immunoglobulins.

Antibodies are soluble glycoproteins (known as globins) that are produced in response to a certain antigen and react specifically with the antigen that stimulated them. There are five major classes of antibody (Ab’s) immunoglobulins (Ig’s). They are IgG, IgM, IgA, IgD, and IgE. Figure 3-1 illustrates an example of IgM immunoglobulin. It has two heavy polypeptide chains and two light polypeptide chains (J chains) bound to a small amount of carbohydrate which makes them a glycoprotein. These chains are linked together by disulfide bonds. In humoral immunity, antibody formation takes place when an antigen is processed by a macrophage, and the antigenic determinants are presented to a T cell. The T cell responds by

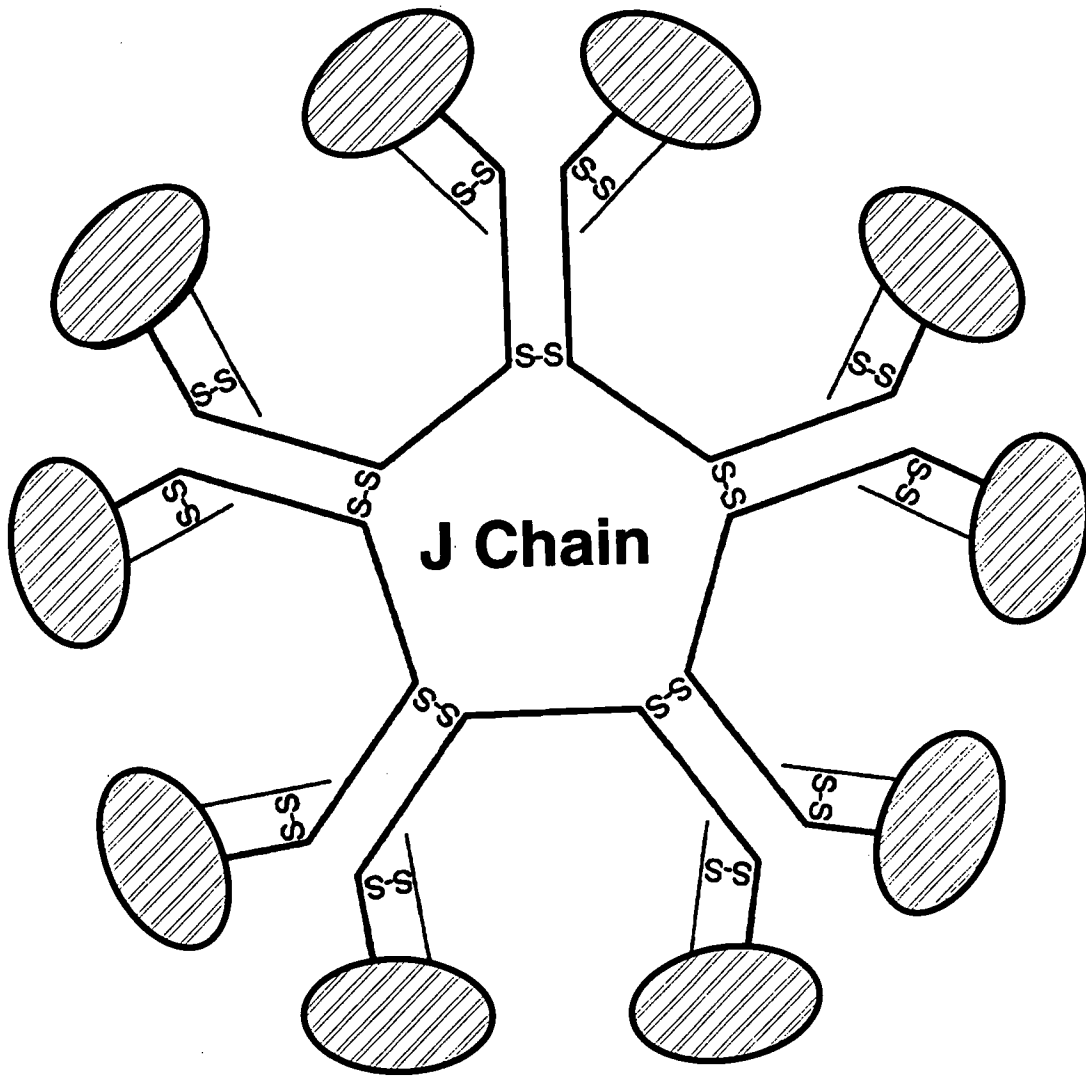


Figure 3-1
Schematic of an antibody.

Immunologic techniques are used to identify antibodies and antigens that are associated with specific diseases in animals, including humans.

Immunologic assays are used to identify antibodies and antigens.

Soluble antigens that react in vitro with homologous antibodies produce a visible reaction called precipitation.

The SRID technique is important in determining the amount of antigen in a solution.

The Mancini method can determine serum immunoglobulin concentration, as well as complement C-reactive protein, alpha-fetoprotein, and transferrin.

producing cytokines (regulator molecules) that cause B cells to produce IgM or become plasma cells. Plasma cells then secrete all types of immunoglobulin.

Various immunological techniques are utilized to identify specific antibodies and antigens associated with different diseases in animals, including humans. One of the more common techniques is the immunologic assay.

Immunologic Assay

Precipitation occurs when a soluble antibody reacts to produce an aggregate that precipitates and becomes visible to the naked eye. Antigens and antibodies must be in optimum portions in order for them to form large lattices necessary for precipitation to take place in gels or solutions. The right dilution of antigen and antibody is necessary for maximum precipitation. Both antigen and antibody must be soluble for precipitation reactions to occur.

Techniques that combine diffusion and precipitation in agarose gel include radial immunodiffusion (RID). Radial immunodiffusion is used to determine the specific concentration of antigens in a patient's serum. It is a quantitative technique that is used to detect and quantitate classes of antibodies or immunoglobulins and other proteins in serum. For example, consider when the antibody IgG is placed in a well and allowed to diffuse in an agarose gel that has a specific known monoclonal antibody (monospecific antiserum) in the gel. When the IgG that serves as an antigen from the patient's serum diffuses and reacts with the monoclonal IgG that is dispersed in the gel, precipitation occurs in a radial precipitin band (see Figure 3-2). In RID, only the antigen diffuses; therefore this represents single radial immunodiffusion (SRID).

Known antigen standards of IgG at low, medium, and high levels are placed into wells at the same time as the patient's serum containing IgG and allowed to diffuse for 48 hours according to the Mancini method. Once the precipitin ring reaches an end point (after 48 hours), each diameter of the precipitin ring is measured in millimeters. The diameter is squared and plotted against the known standard IgG concentrations in milligrams per deciliter (mg/dL). The higher the concentration of IgG (that is functioning as an antigen), the greater the diameter of the precipitin ring (see Figure 3-2).

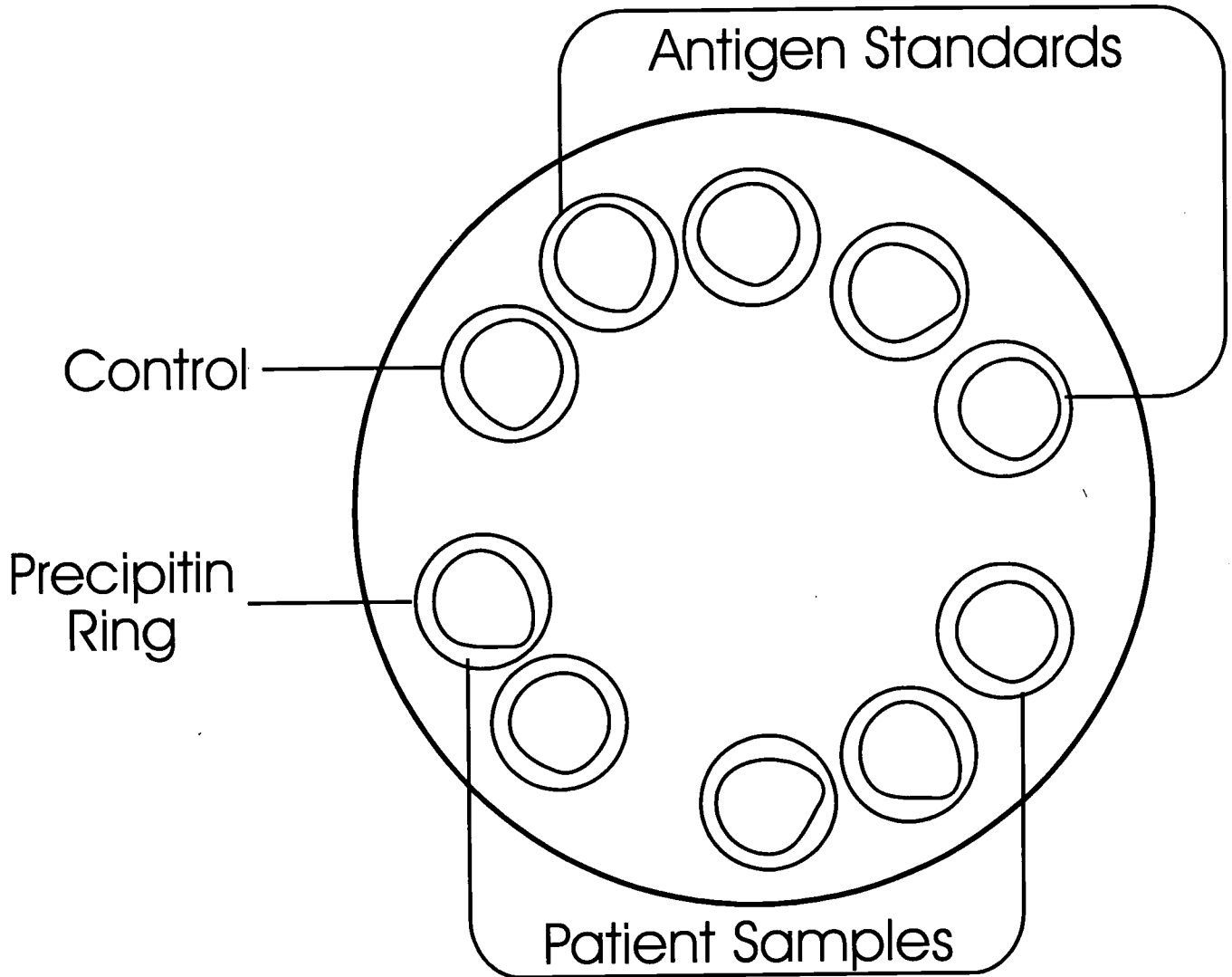


Figure 3-2
Radial immunodiffusion.

Other techniques include the Ouchterlony technique. In this technique, the antigen and antibody both diffuse in the agarose gel. Therefore, it is called double radial immunodiffusion (DRID). This procedure allows for identification of antigens and antibodies. It identifies unknown proteins that are soluble in a gel. When identifying an unknown antigen, a known identical antigen is placed in a well across from the unknown. The known specific antiserum (SA) is placed in a well between and below the two antigens, and diffusion takes place in the gel. If the antigens are the same, a reaction of identity results in precipitin bands that join together about half way between the antiserum well and the antigen wells. If a reaction of nonidentity results, then the two precipitin bands will develop and cross. A third reaction can occur that results in partial identity when one of the bands overlaps (see Figure 3-3). The double diffusion Ouchterlony technique can be used to identify proteins linked to cancer or viruses. Methods that employ movement of charged particles (proteins) in an electrical field alone or in combination with diffusion include electrophoresis and agglutination.

The Ouchterlony method can be used to determine whether an unknown substance is the same as a known substance (e.g., cancer cells or viruses).

Electrophoresis is the movement of charged proteins (antigens) or particles in an electric field (see Chapter 1). When human serum proteins are spotted and allowed to be exposed to electrophoresis at the proper pH and ionic strength in buffer, the serum proteins separate into bands on cellulose (acetate paper) creating a typical pattern based on the weight and charge of the proteins in serum.

The separated antigens or protein bands can be stained with a special blue dye to visualize the various serum proteins. Each serum specimen is compared to a serum control electrophoretic pattern to determine if any abnormalities occur in the test serum (from the patient). This part of the electrophoretic test is qualitative; however, a scanning spectrophotometer is then utilized to quantitate each serum protein to determine the existence of human disease (see Figure 3-4).

Immunolectrophoresis

Immunolectrophoresis (IEP) employs electrophoresis diffusion and precipitation. It provides a means for separating a mixture of antigens and comparing them to known antigens with known antiserum (antibodies). IEP allows for the identifi-

Immunolectrophoresis combines electrophoresis with double gel diffusion to identify the number of antigens producing antibodies and their relative amounts.

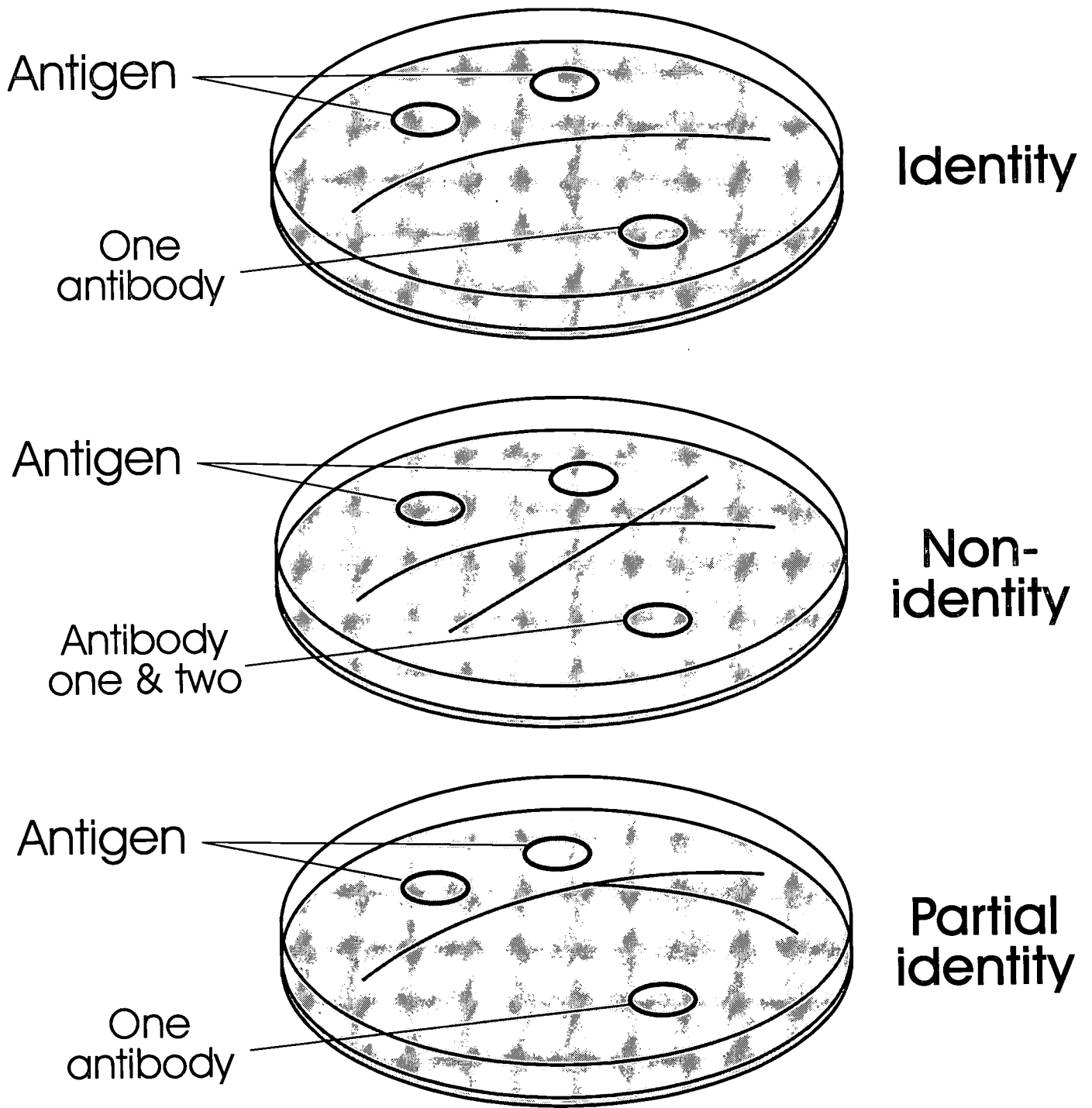
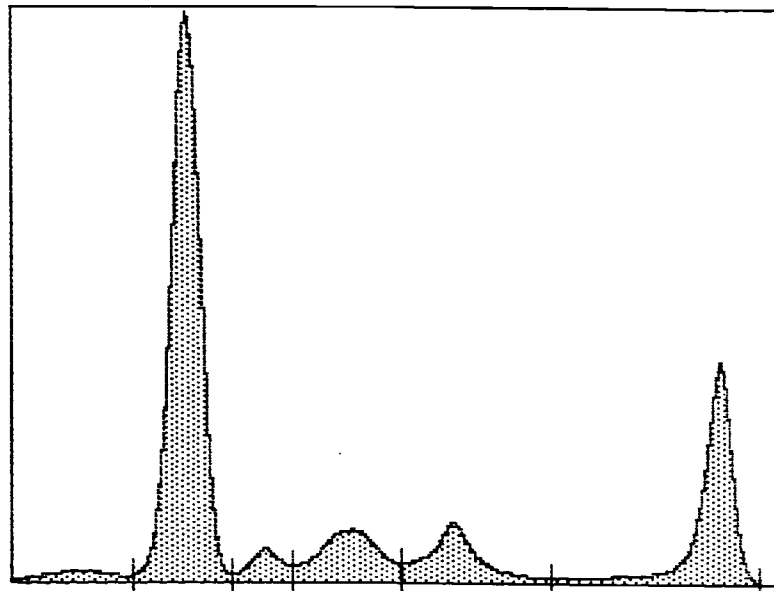


Figure 3-3
Ouchterlony double diffusion.



Fraction	Rel%	G/dL
Albumin	54.3 -	3.75
Alpha 1	3.6	0.25
Alpha 2	10.1	0.70
Beta	9.8	0.68
Gamma	22.2 +++	1.53

Total G/dL 6.90 A/G: 1.19

Comments:

Figure 3-4
Electrophoretic pattern.

cation of the number of antibodies and their amounts. A variant of this procedure is crossed immunoelectrophoresis. This procedure is more sensitive than IEP and allows for precise measurement of the amount of antigen present in a substance.

Agglutination

Agglutination is the visible clumping of red blood cells or any particle, used as an indication of a specific antigen-antibody reaction.

Agglutination reactions are useful for typing blood, determining antibody titer, and identifying microbes.

Agglutination reactions are used to identify microorganisms (such as bacteria, viruses, and fungi) and to type blood. In agglutination reactions, the antigens are particulate (non-soluble) while the antibody is soluble in the solution. The antigen can be a whole cell or part of a cell. An antigen is called an *agglutino-gen* while an antibody is known as an *agglutinin* in agglutination reactions.

In an agglutination test that takes place on a glass slide, one or two drops of antigen are placed on the slide along with a drop of antibody or antiserum. They are then mixed together and clumping takes place within one to three minutes when the antigen combines with the known specific antibody. Standard positive and negative controls are done at the same time as the test sample or specimen (see Figure 3-5).

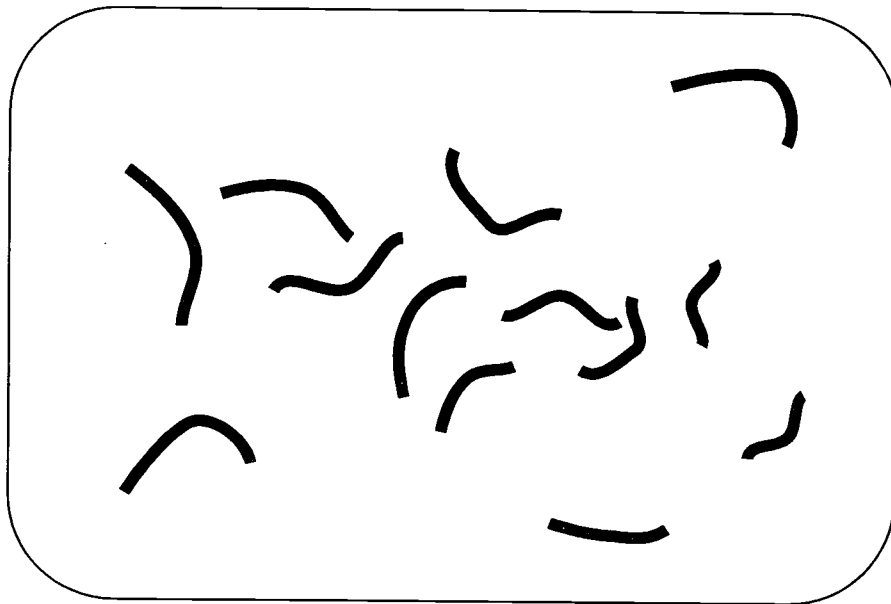
Direct agglutination occurs when *Escherichia coli* OH137 known antiserum is utilized to identify whole cells of *E. coli* OH137 (antigen). Passive agglutination slide tests occurs when a carrier such as latex adheres to an antigen that is employed to identify an unknown antibody.

Tube agglutination serological tests are performed to determine the antibody titer against a particular microbe or bacterium that causes disease such as *Francisella tularensis* (causes undulant fever). The antibody titer is determined only after a positive slide agglutination screening test. Antibody titer is defined as the reciprocal of the highest dilution of the antibody that will cause an agglutination reaction. It is utilized in diagnosis of many microbial diseases including tularemia.

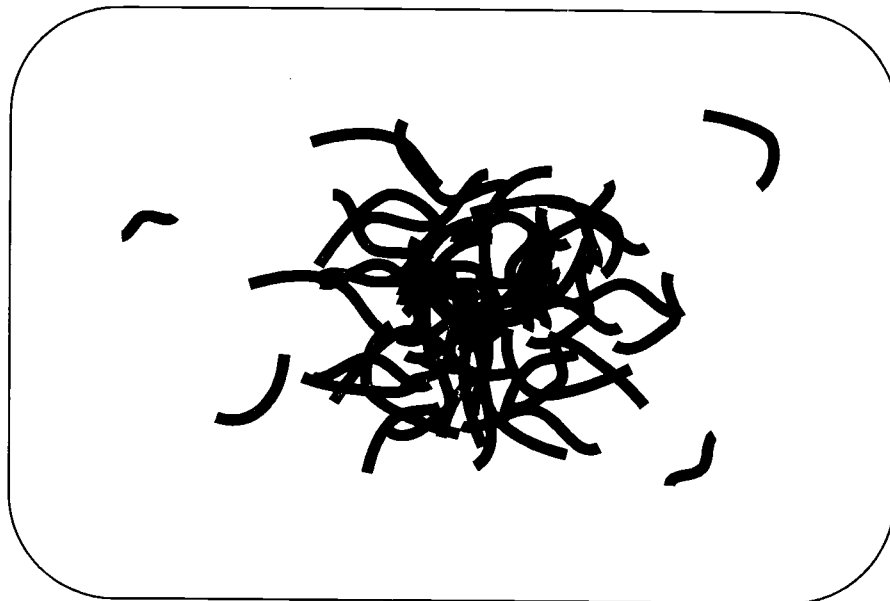
Antibody titer is the lowest dilution of antibody that results in agglutination.

Hemagglutination results when clumping of red blood cells takes place on glass slides, in micro wells, and on microtiter plates. Hemagglutination is used to identify blood types and screen for the presence of certain viruses. Some viruses, such as influenza virus, will adhere to guinea pig red blood cells and cause cross-linking that results in a hemagglutination reaction.

E. coli



No
Antiserum



With
Antiserum

Figure 3-5
Agglutination of *Escherichia coli*.

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA is an immunoassay used to detect antigens or antibodies in human serum. Hepatitis, HIV infection, and rubella are examples of disorders that can be diagnosed with the ELISA.

The ELISA is used to detect antigens and antibodies in very small concentrations. This is one of the most widely employed immunological techniques. This technique may be performed in a test tube, microliter plate or on plastic beads with very limited amounts of reagents.

The liquid phase ELISA is employed in plastic microtiter plates and test tubes. The solid phase technique occurs on a ColorPac membrane. There are two types of ELISA: (1) direct and (2) indirect. The double antibody sandwich method (direct) is used to detect antigens (see Figure 3-6). In this method, known antibody is placed in unknown serum. A known antigen is added and allowed to react with the antibody. Then an antibody bound to an enzyme is added and allowed to react with antigen bound to the initial antibody. After washing takes place, a substrate is added and the enzyme will react, resulting in a change in substrate color. If the antigen reacts with antibody in the first step, a positive test will result. An ELISA reader optical density scanner is utilized to detect and quantify the colored compound.

Agglutination results when a homologous antibody reacts with a particulate antigen.

The indirect technique is used to identify antibodies. An antigen is first bound to the plastic well walls; then patient serum containing possible antibodies is added. If specific antibodies bind to the known antigen, the antibody is not washed out of the well. An antihuman globulin (antibody) attached to an enzyme is added. After a reaction by the antihuman globulin enzyme complex, a colored compound results from action of the enzyme after the addition of substrate.

Nephelometry

Nephelometry is the measurement of light scatter produced by particles suspended in a solution.

Nephelometry is replacing radial immunodiffusion (RID) for determining immunoglobulins in the serum, as well as many other important proteins that can be correlated with different diseases in man. This method also detects antigens in human body fluids. Antigen and antibody complexes that result in solution due to antibody-antigen reaction have the ability to scatter light. The light that is scattered by the immune complexes is detected and measured as light scattering intensity units against the known concentrations of standard antibody or other proteins in mg/dl.

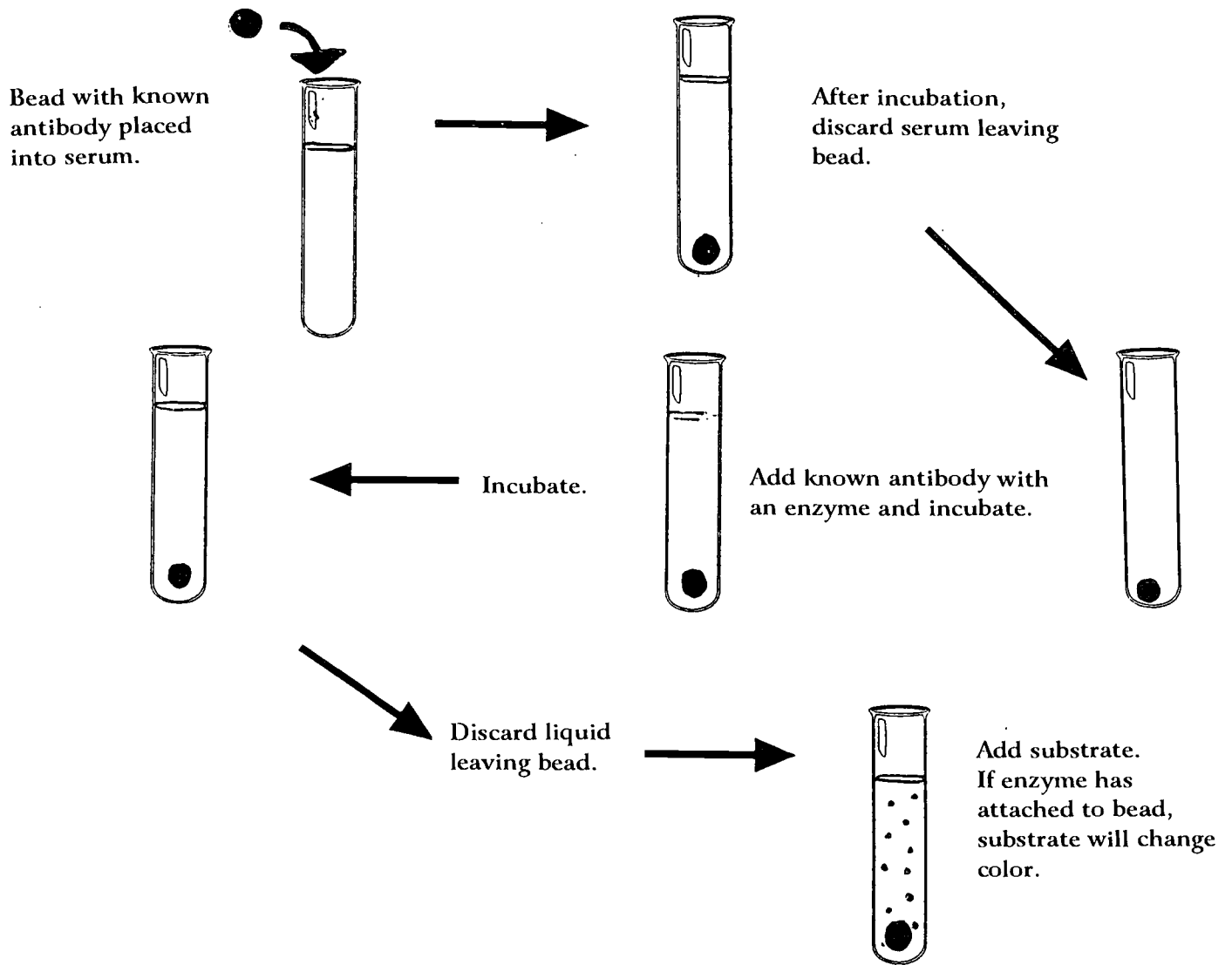


Figure 3-6
Enzyme-linked immunosorbent assay.

The specimen (serum containing antibodies and other proteins) is always compared to three standard concentrations of brown proteins (Ag's) or antibodies for quantification. When the Behring Nephelometer methodology is employed, latex agglutination is utilized if antigen-antibody reactions do not produce immune complexes that are large enough to detect for measurement. Latex particles adhere to antigens that allow agglutinations to take place (that will form measurable immune complexes).

Besides measurement of antibodies, other important proteins in serum, such as complement C₃, can be quantified with known specific antibody to C₃. Low levels of C₃ in man indicate a weakened ability to kill bacteria and make a person more susceptible to disease-causing microbes.

Countercurrent Electrophoresis (CE)

Countercurrent electrophoresis is used to identify antigens (microbes), such as fungi or bacteria, that cause disease in man. It is also useful in identifying antibodies in serum that are correlated with some autoimmune diseases.

This method (CE) is utilized only when antigens are negatively charged and antibodies are positively charged. The antigens and antibodies are placed in wells that have been cut out in agarose gel having an alkaline pH. Electrophoresis of these antigens and antibodies takes place that allows for movement toward each other. When the antigens and antibodies reach optimum proportions as an electrical current is passed through them, a precipitin band forms if the antibody is specific for the antigen in question. Known antigen and antibody controls are run at the same time as the patient's specimen. This technique allows for a more rapid precipitation reaction to take place between the antigen and antibody (in about 45 minutes) allowing quick identification of disease-causing microbes.

Radioimmunoassay (RIA)

This assay is used to detect and measure very small concentrations of antigens, haptens, and other molecules such as insulin or certain drugs. Radioimmunoassay is based on competitive binding between an unknown nonradioactive antigen and a known radiolabeled antigen for the same active sites on the specific antibody.

RIA is an immunoassay reaction in which the labeling molecule is a radioisotope.

A known amount of radiolabeled antigen is mixed with an unknown amount of antigen, along with a limiting amount of rabbit antiserum (to antigen). The antigens and antibodies react to form antigen-antibody complexes that are soluble. Once the complexes are formed, goat antiserum (antibody) to rabbit antigen is added, and a large complex is formed and precipitates. The precipitated complex containing the unknown antigen is then measured in counts per minute (cpm) for radioactivity. The higher the mg/dl concentration of the unknown antigen, the less radioactivity (in cpm) is detected. Three or four different standard concentrations of the unknown antigen are used to construct a standard curve (see Figure 3-7).

An advantage of RIA is its high sensitivity and resistance to interference in the assay environment. However, numerous disadvantages, such as cost of disposal of waste, the requirement for licensing to perform the test, short reagent shelf-life, and the addition of a separation step, have encouraged clinicians to use alternative immunoassays. These include nonradiolabeled assays, chemoluminescent precursors, and fluorescence molecules.

Fluorescent Antibody Technique (FAT)

Antibodies against insoluble or particulate antigens can be detected with FAT.

There are two FATs that can be utilized to identify antigens and antibodies. The direct FAT is mainly employed to identify antigens while the second FAT (referred to as indirect FAT) identifies serum antibodies. A special fluorescent microscope is needed to see fluorescent antigens or antigen-antibody complexes that have a fluorochrome or dye attached to an antihuman globulin. The antigen-antibody complex is irradiated with UV light that is absorbed by the dye. Fluorescence occurs when the dye transmits light at certain wavelengths that allows viewing of the fluorescence antigen-antibody complexes.

Direct fluorescent antibody technique involves use of a known antibody linked to a dye. The unknown antigen is placed on a glass slide and fixed. The known specific antibody linked to the fluorochrome is added to the slide. If the antigen is specific for the antibody, fluorescence occurs. If the antigen is not specific for the antibody, no fluorescence takes place because the fluorochrome was washed off the slide. Indirect fluorescent antibody technique is a two-step procedure. Known antigen is attached to the slide, and unknown serum antibody is added and allowed to react with the antigen. If the antibody is

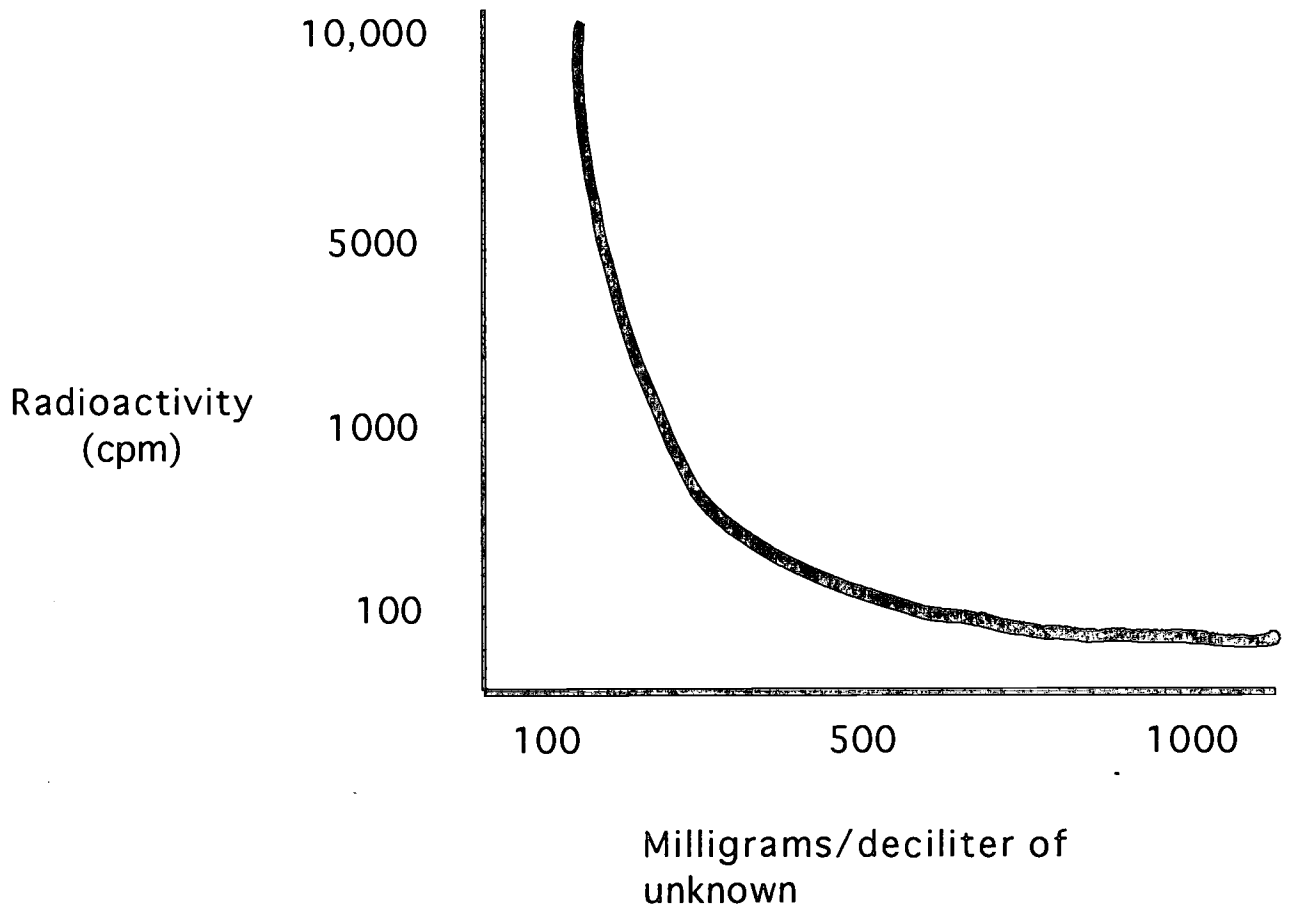


Figure 3-7
Radioimmunoassay (RIA) curve.

specific for the antigen, an initial antibody complex results. An antihuman globulin linked to a fluorochrome is allowed to react with the initial antigen-antibody complex. If the serum antibody is specific for the antigen, a positive test results for the serum antibody in question (that causes fluorescence), while a negative test results in no fluorescence.

In summary, modern analysis of antigen-antibody complexes and identification of different antigens and antibodies, as well as other plasma proteins, have resulted from new technological innovations in instrumentation. Newer immunologic techniques allow for greater sensitivity in identification of unknown substances.

Recent immunologic procedures that utilize instrumentation and computers linked with techniques such as ELISA, RIA, and adaptation of nephelometry for replacement of RID, as well as other methods, such as DNA probes and the polymerase chain reaction, PCR (see Chapter 15), will continue to open new horizons for identification and quantification of antigens, antibodies, and other proteins.

Questions Commonly Asked by Students

Question 1: What type of antigen-antibody reaction occurs when both the antigen and antibody are soluble in the medium?

Precipitation.

Question 2: What immunologic technique can be utilized to identify protein in redfish employing the specific types of antigen-antibody reaction in the above question?

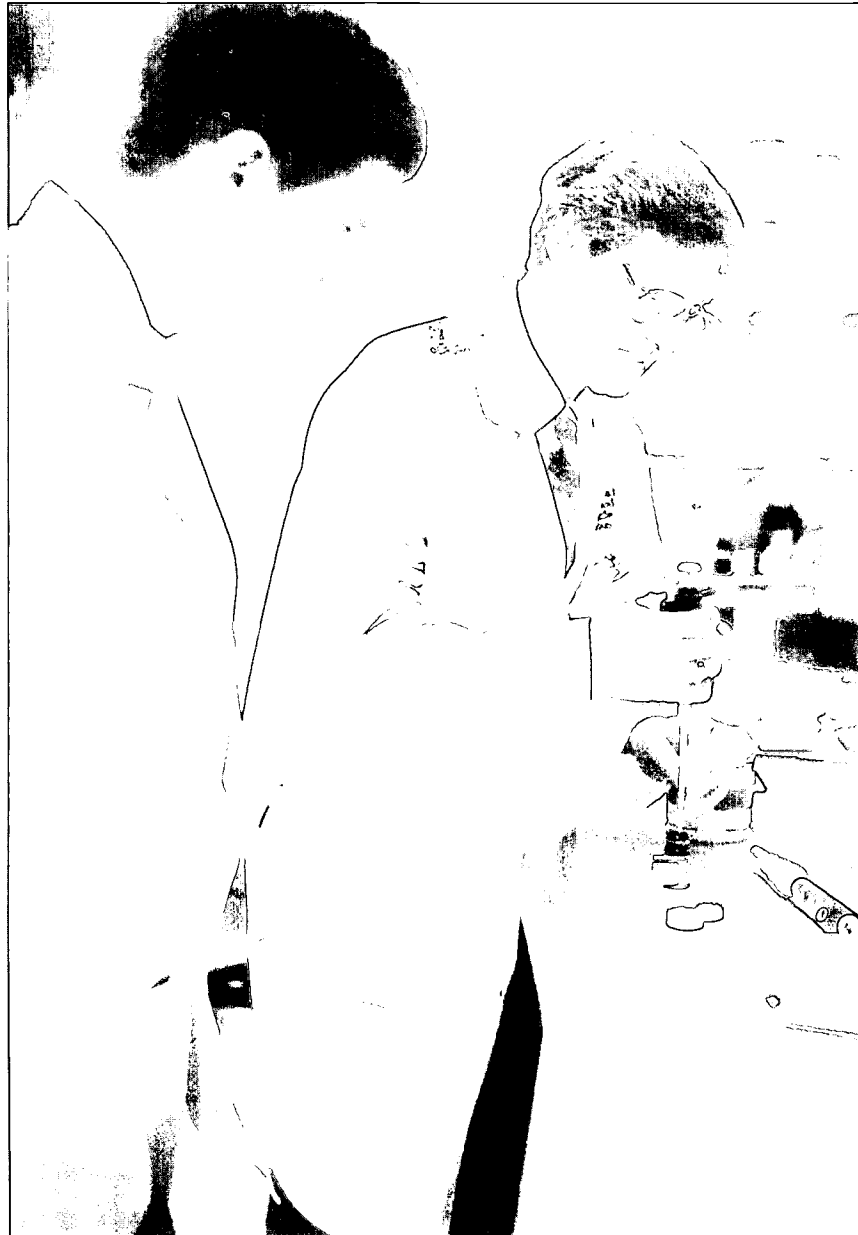
The Ouchterlony techniques could be used.

Question 3: What three types of reactions could occur with the Ouchterlony techniques?

Identity, nonidentity, and partial identity.

Question 4: To identify a disease-causing bacterium like *E. coli* in meat, what test could be used to identify the antigen-antibody reaction?

Agglutination.



Dr. Selvestion James demonstrates how to detect antibodies in a patient's serum with a card test rotator to Mike Morris, a senior cardiopulmonary science student at LSU Medical Center, School of Allied Health Professions, in Shreveport, LA.

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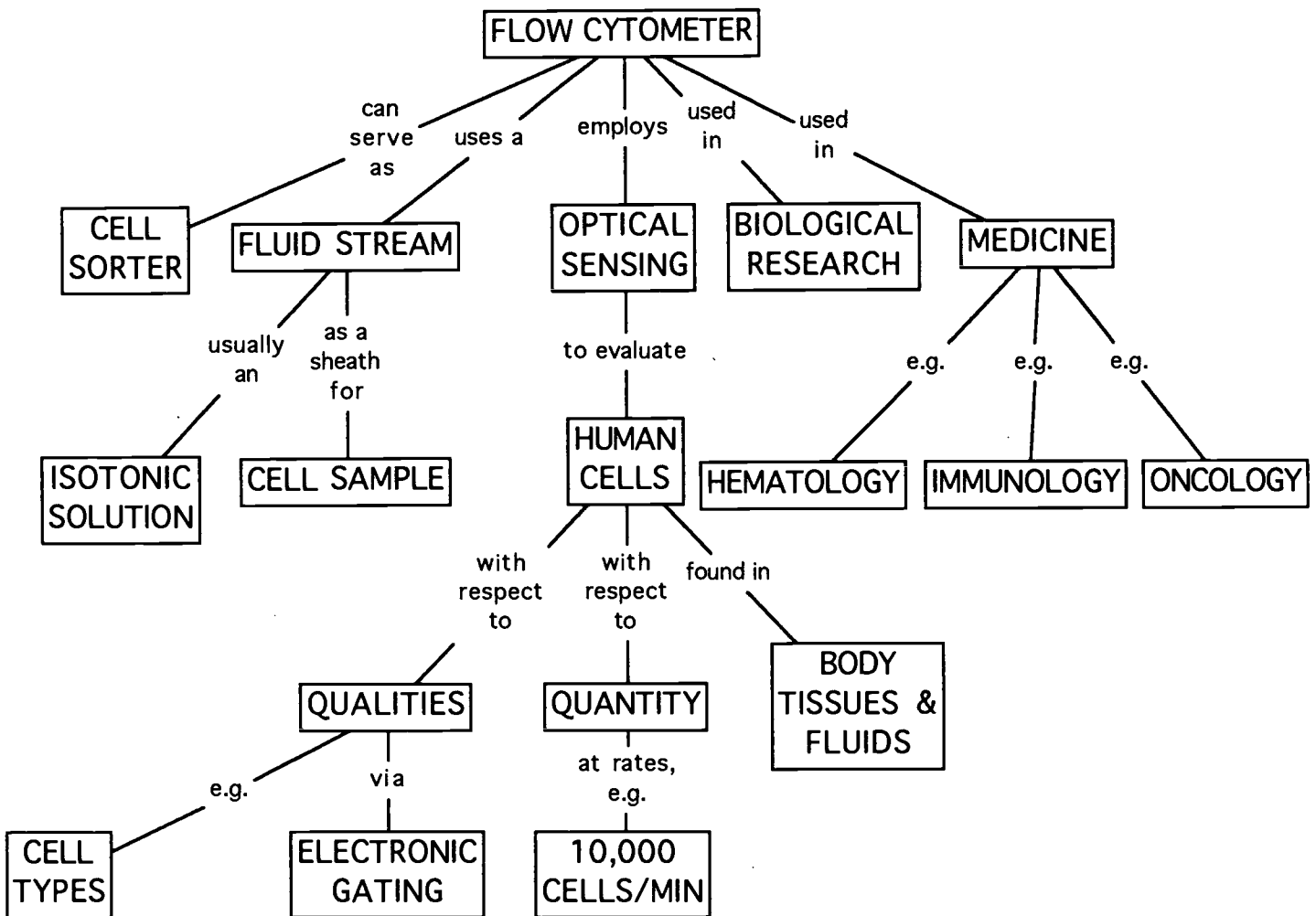
FLOW CYTOMETRY

4

Bridget L. Langley

Overview

1. Basic components of a flow cytometer
2. Sample preparation
3. Limitations of the flow cytometer



Flow Cytometry

Flow cytometry measures properties of cells and cell organelles that are suspended in a liquid medium.

An exciting new alternative to traditional microscopic analysis of cells is flow cytometry. The name of the instrument is descriptive of its function: a *cytometer* is an instrument that measures cells, and *flow* describes the method by which these cells travel through the machine. In use since the 1950s, the flow cytometer was, at first, primarily a research tool. Today, however, most large clinical and diagnostic laboratories have added at least one to their instrument array. They are also very common in a variety of industrial settings.

A flow cytometer provides the user with an automated method for evaluating cells, both quantitatively and qualitatively. The cells can be measured either by electronic or optical means and, in contrast to traditional microscopic analysis, an objective rather than subjective measurement is obtained. An additional advantage of the flow cytometer is that it provides results rapidly. Most instruments have the capability of examining as many as 10,000 cells per second, an analysis rate that far exceeds that of even the best trained clinical laboratory scientist.

In the optical sensing of cells by flow cytometer, cells in suspension are analyzed individually as they pass single file through a light beam. As each cell enters the beam, it interrupts and scatters the light. Measurements of these light deviations are recorded and plotted by a computer. The resulting information, or *histogram*, is a detailed graph that provides the user with a profile of the cells in the original sample.

The Basic Flow Cytometer

The four main components of a flow cytometer are a fluid stream within which cells are transported, a laser light source, a detector system, and a computer (see Figure 4-1). The sophistication of both the individual components and the machine itself varies, depending on the user's requirements. Flow cytometers in which individual components can be altered to fit different research projects are also available.

Four basic components of the flow cytometer include:

- 1) fluid stream*
- 2) laser light source*
- 3) detector system*
- 4) computer.*

The sample being studied is first injected into the instrument's flow chamber, where it becomes encased in a liquid stream, usually an isotonic fluid such as saline. The surrounding fluid is known as a *sheath*. Cells are aligned single file precisely within the center of the stream, a process known as

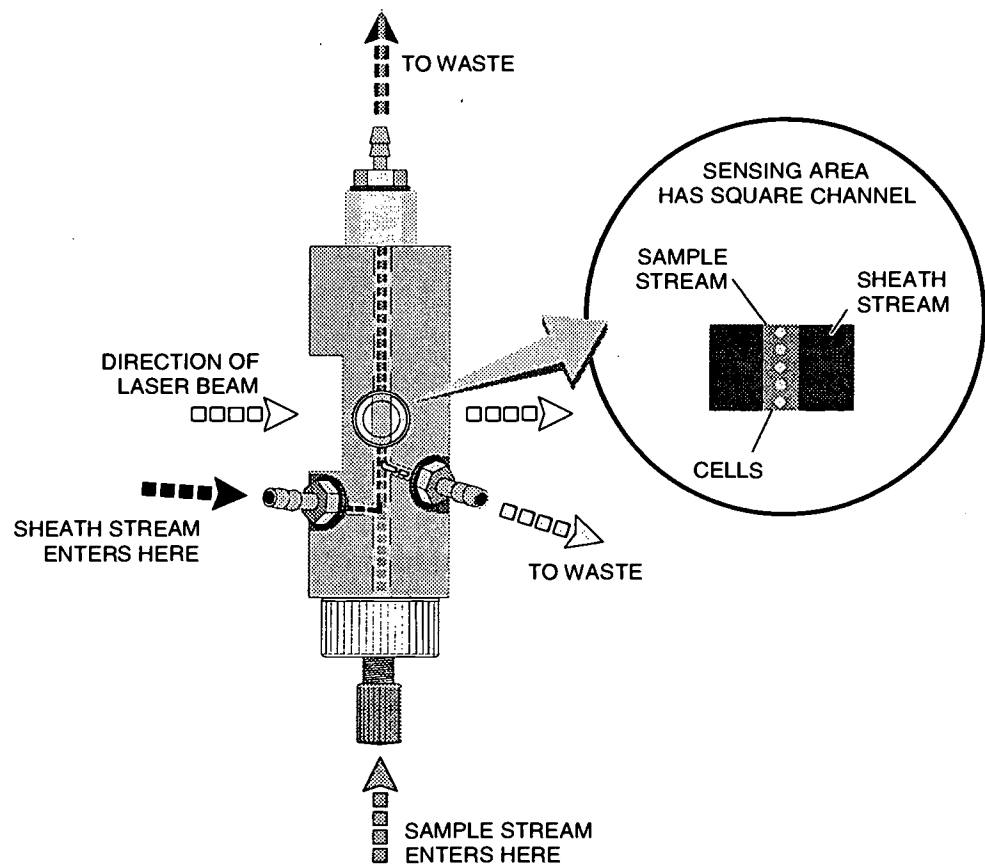


Figure 4-1
The basic components of a flow cytometer.

Laminar flow of the liquid stream allows for the measurement of single cells.

Lasers commonly used are:

- 1) argon
- 2) krypton
- 3) helium-neon
- 4) helium-cadmium.

Light scattering refers to the ability of cells to scatter light.

hydrodynamic focusing or *laminar flow*, which ensures that each cell will receive maximal laser light exposure. The difference in pressure between the sample and the surrounding fluid maintains the single file configuration of the cells as they flow through the detectors.

Once the sample stream leaves the flow chamber, it passes through a specifically sized orifice. Next, cells pass between a light source and a light detector. While the light source is usually a laser, a less expensive, less powerful alternative is a mercury arc light. Lasers are preferable, as they provide a stable, powerful source of monochromatic light. The argon gas laser is the most common type of laser used. It produces light that has a wavelength of 488 nanometers. Many fluorescent dyes added to the cell suspension in flow cytometry are excited by this wavelength, making this a particularly desirable type of laser. Other examples of light sources include krypton, helium-neon, and helium-cadmium lasers.

As each cell enters the light beam, it interrupts and scatters the laser light. Interruptions in the light beam correlate with the number of cells passing through the orifice. Particles scatter light in all directions. Forward-angle light scatter (FALS) is that which is scattered 2-10 degrees. This type of scatter relates to the size or volume of the cell. The larger the cell, the more light scattered (see Figure 4-2).

The light scattered at 90 degrees (90° LS) relates to the internal structure of the cell. Orthogonal, right-angle, or wide-angle are other terms used to describe this type of scatter. The more granular a cell, the more light it will scatter in this direction. For example, the three main types of white blood cells, or leukocytes, can be separated based on their 90° LS. Lymphocytes, the least granular of the three, have the lowest 90° LS; then monocytes, followed by granulocytes, which are the most granular (see Figure 4-3).

By combining the results of the FALS and the 90° LS with the number of particles sensed in each category, a three-dimensional histogram can be generated by the computer (see Figure 4-4). Once the user has a general idea of the various cell populations present in the sample, a more detailed analysis is possible. For example, an electronic *gate* can be drawn around a selected cell group and the characteristics of this subset displayed on a separate histogram (see Figure 4-5).

For particles larger than 20 micrometers, a neutral density filter may be inserted in the light path to reduce the amount of light and the pulse height.

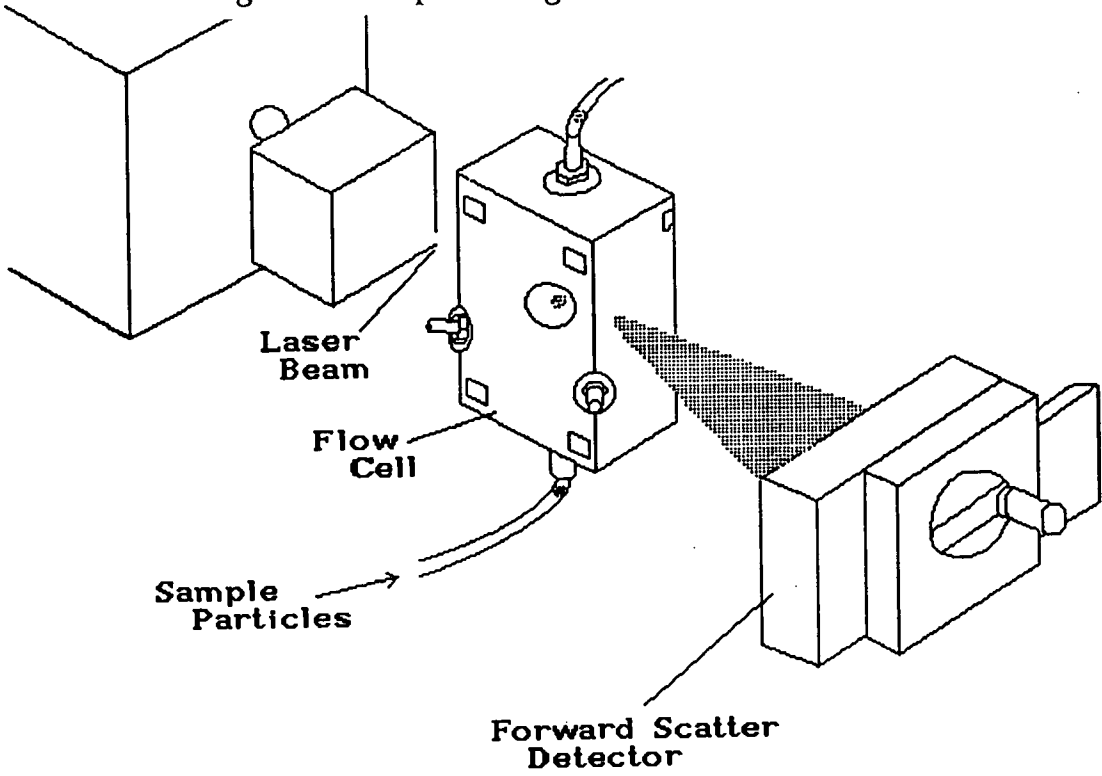


Figure 4-2
Measurement of FALS (forward-angle light scatter).

Besides size, granularity is also detected. Laser light deflected off internal structures or granules within a particle supplies the majority of the side scatter light.

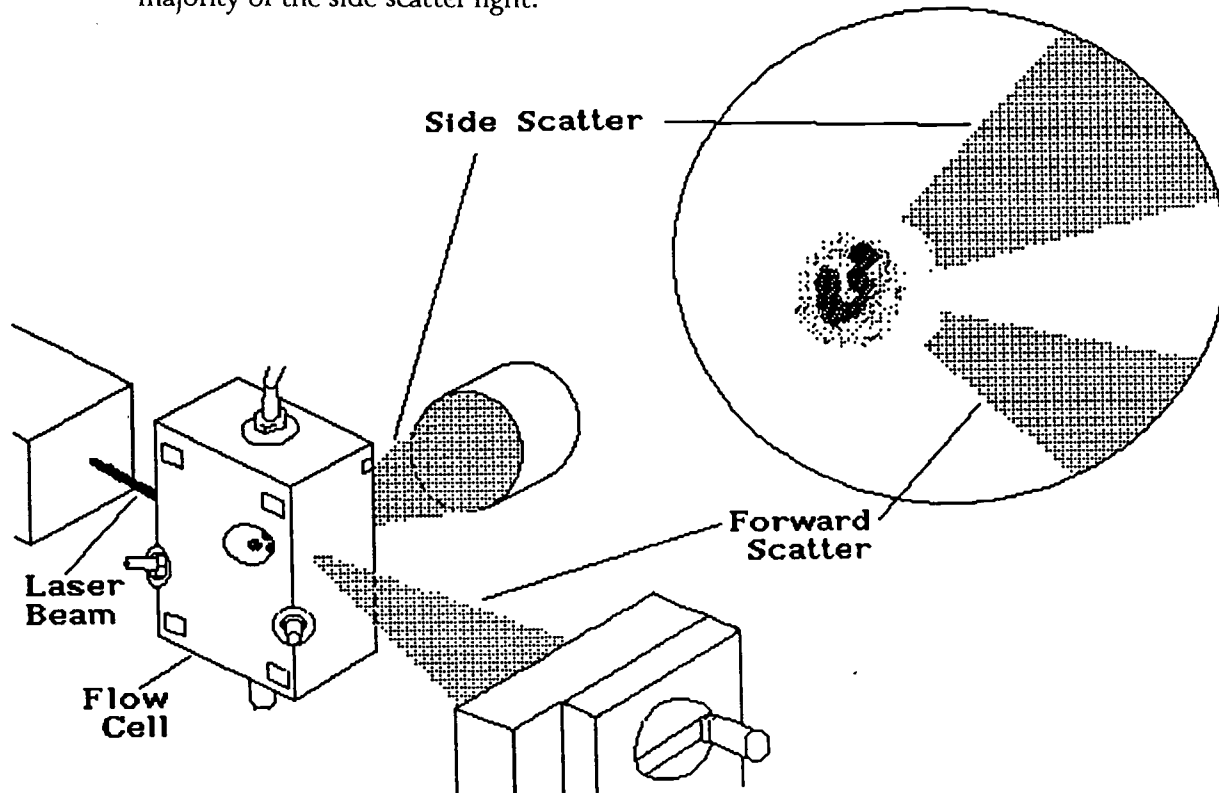


Figure 4-3
Measurement of 90° LS (light scatter).

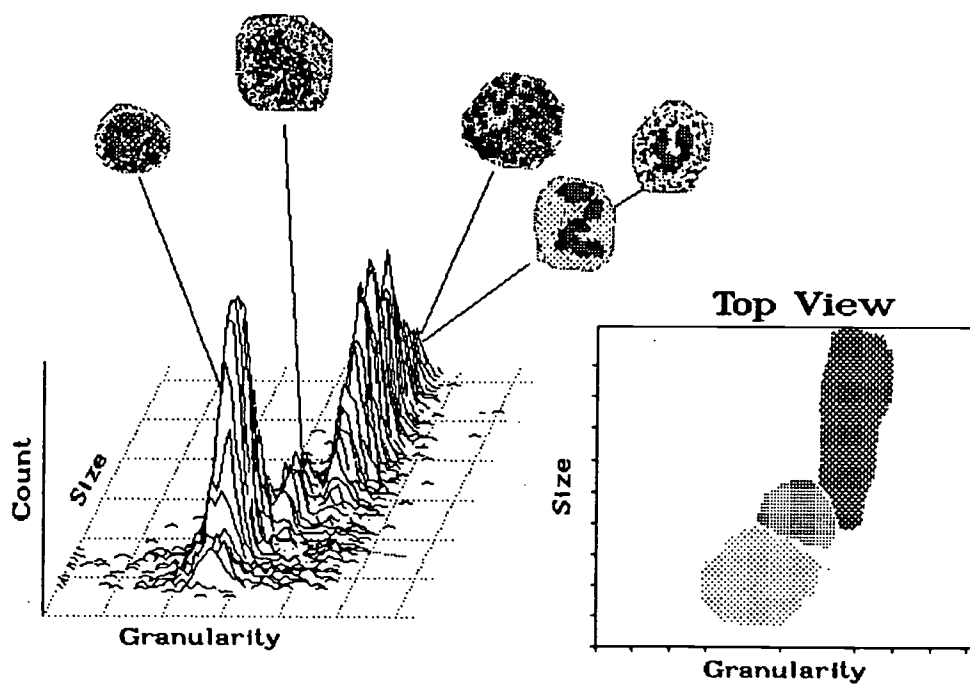


Figure 4-4
Combination of FALS and 90° LS.

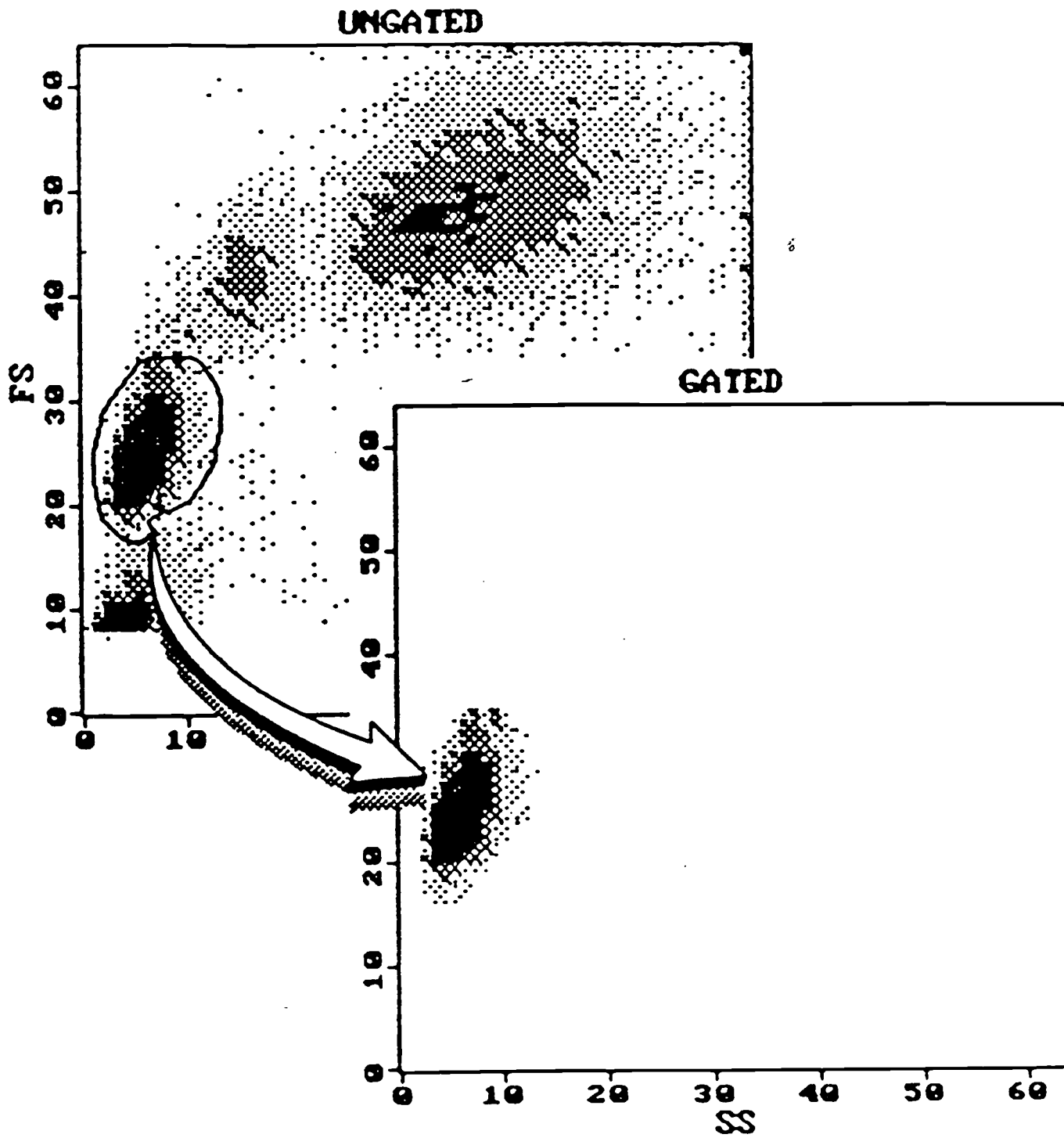


Figure 4-5
Electronic gating to show subset's characteristics.

Fluorochromes are molecules used to tag cells that can absorb and re-emit light as fluorescence.

For additional information and characterization of cells, the operator can add fluorescent dyes to the initial cell suspension. The dyes, or *fluorochromes*, are selected on the particular cell component with which they bind, such as cellular DNA or RNA, or various intracellular enzymes. Dyes commonly used are acridine orange, fluorescein-isothiocyanate (FITC), and phycoerythrin (PE).

Dichroic mirrors split a light beam and direct it into two different directions.

Even more sophisticated measurements are possible by using a combination of dyes, such as PE *and* FITC. As fluorescently labeled cells pass through the laser, their fluorescence is measured simultaneously with FALS and 90° LS. Optical filters and dichroic mirrors within the instrument separate the multicolored light and direct it to the appropriate photomultiplier tube (PMT). Photomultiplier tubes are devices that convert low-level light energy to electrical energy. For example, one PMT may collect the green fluorescence of fluorescein, while another only receives the red-orange light of phycoerythrin. Characterization of dual populations of cells is then possible based on which dye they bind. A series of detectors and photomultiplier tubes picks up the scattering and emitted fluorescence and converts the information to analog signals that are then digitized. The computer is essential at this stage, as it processes all light scatter information, providing the user with a detailed analysis of the sample. The computer also serves as a data storage bank and is responsible for operating the instrument.

Flow cytometry can also sort and remove cells from a liquid suspension.

Beyond counting and characterizing cell populations, some flow cytometers can also serve as cell sorters. That is, a targeted population of cells can be physically separated from the sample, and a pure sample of such cells is then available for further studies. To separate a desired population of cells, the computer sends a message to a charging collar to change the charge on the stream when it contains the desired cells. Deflection plates then send the cell to the appropriate collection receptacles based on the charge. That is, positively charged cells will be attracted to the negatively charged plate, negatively charged cells to the positive plate (see Figure 4-6).

Sample Preparation

Body tissues can be analyzed by flow cytometry after being broken down by enzymes or other techniques.

Both body fluids and tissues can be analyzed with a flow cytometer. Peripheral blood samples are ideal, as the cells are already in suspension. In the case of tissues, the specimen must first be broken down in order to flow through the instrument.

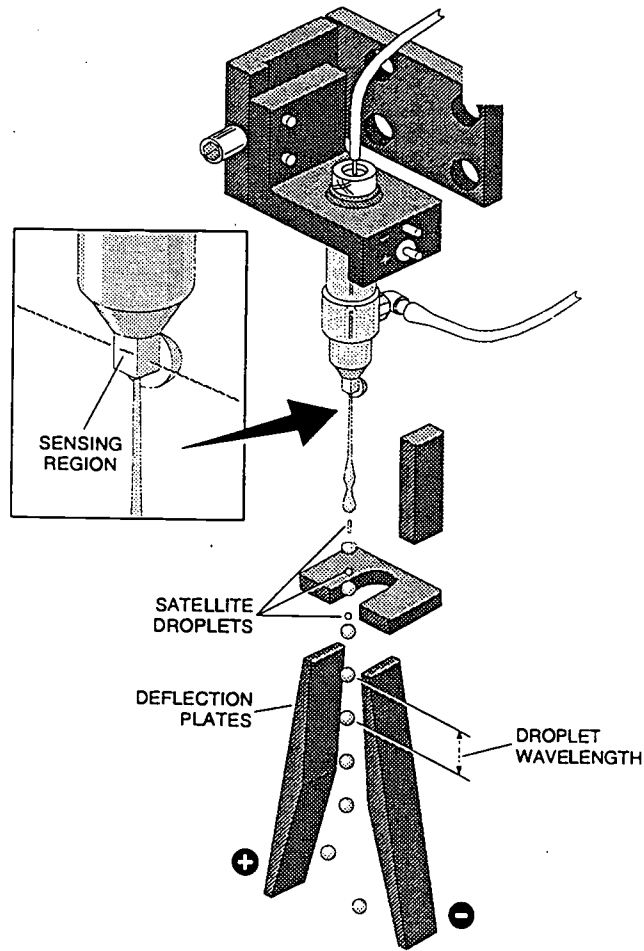


Figure 4-6
Diagram of a cell sorter.

For analysis, the user needs single, intact cells in a fluid suspension. Tissue samples, such as skin biopsies, can be disaggregated by enzymatic digestion or various other techniques. However, this can sometimes present a problem because the breakdown of tissue may yield unwanted artifacts.

The uses of the flow cytometer are extremely diverse, ranging from straightforward clinical applications to inventive industrial research projects. The instrument is used in hospital laboratories for diagnostic and prognostic purposes and on board ships for studies in the field of marine biology. In hospital and clinical settings, the three main areas of use are in the hematology, immunology, and oncology departments. In hematology, the department responsible for studying the morphology of blood and blood forming tissues, these instruments are used to characterize the various red and white blood cells. The flow cytometer is capable of providing the clinician with data regarding cell size and cytoplasmic granule content. This can lead to a cellular profile, or differential, which can be used to diagnose blood disorders, such as leukemia.

Many hematology laboratories also use flow cytometers to perform reticulocyte counts. Reticulocytes are immature erythrocytes, or red blood cells. In cases where the body increases red cell production, such as in anemia, the reticulocyte count goes up. These immature cells contain residual amounts of RNA; whereas, fully mature erythrocytes do not. By using a fluorescent dye that binds to the RNA, the clinical laboratory scientist is able to assess red cell production.

Immunologic analysis of cells is of benefit in the diagnosis and treatment of a multitude of inherited and acquired immune disorders. A common application is the characterization of the specific subsets of white blood cells that are responsible for the overall control of the immune response. With this information, for example, disease progression in a patient with acquired immune deficiency syndrome (AIDS) can be monitored. Effectiveness of drug therapy in patients can also be measured in this same manner.

The method by which such cell population characterization is performed involves the use of reagents known as monoclonal antibodies. These compounds are designed to bind specifically with a known cell surface protein. For example, a type of white blood cell, a helper T cell, has a protein known as CD4 on its surface. If the monoclonal antibody attaches to the cell,

Monoclonal antibodies are compounds designed to bind specifically to unknown cell surface antigens.

antibody attaches to the cell, then that cell can be definitively labeled as a helper T cell. For flow cytometric applications, the monoclonal antibody is first labeled with one of the fluorescent dyes; when it attaches to the cell, that cell then fluoresces, an event which is detected as the cell passes through the laser beam. Fluorescence is recorded and displayed on a histogram, allowing determination of the number of helper T cells in a sample.

Another rapidly expanding application in this area is in the field of transplantation. The compatibility of a donor organ with the recipient's immune system can be measured. In addition to solid organ transplantation, such as kidneys, livers, and hearts, the flow cytometer is also being used by bone marrow transplant programs. Once the transplant has been performed, the flow cytometer evaluates the recipient's immune system as it responds to the presence of the foreign organ. The physician can then make any necessary changes in the patient's immunosuppressive medications, based on the results.

Flow cytometers are used by oncologists to study tumors. Results can be used to diagnose cancer, provide prognostic information, and monitor therapy. Much of the information in this area comes from cell cycle analysis, which involves examining how the DNA content of a sample changes during cell growth. The DNA content of malignant cells is different from that of normal, or healthy, cells. For this type of analysis, the operator must select a dye that will bind to DNA, such as propidium iodide. The amount of fluorescence detected is related to DNA content. The resulting sample information is compared with known normal values to provide the clinician with diagnostic information.

Related to DNA analysis is chromosomal analysis, or karyotyping. The DNA content of individual chromosomes can be measured, providing information about fetal sex and, in some cases, chromosomal aberrations. Results from such analysis can be used by fertility laboratories for prenatal screening and genetic counseling.

Research labs frequently rely on the cell sorting capabilities of the instrument to provide them with a purified cell preparation. These cells, once separated, can then be grown in culture for further studies, placed directly on a slide for morphologic analysis, or sent back through the machine with different fluorescent markers to further characterize the cells morphologically or biochemically.

Since DNA content of cancer cells differs from DNA content of normal cells, flow cytometry can be used in the diagnosis of cancers.

Chromosomal karyotyping involves staining cells in the metaphase, lysing, and evaluating them.

Limitations of the Flow Cytometer

For some laboratories, the main limitation of the flow cytometer is its cost and the amount of expertise required to run the instrument. However, advances in laser technology have greatly impacted the field of flow cytometry. Many of the larger, water-cooled lasers have been replaced with smaller, less expensive air-cooled lasers, allowing the resulting instrument to fit on most laboratory benches.

Currently, the flow cytometer requires a great deal of technical expertise to operate. Although the instrument is becoming more user-friendly, it requires a great deal of experience both to select the appropriate tests to run and then to interpret the results. Most instrument manufacturers will help to train the user when an institution purchases one of its machines. Formal courses and workshops are also available for more advanced training.

Advancements in both computer technology and the field of immunology will have tremendous effect on the future of the flow cytometer. As more cell surface markers are identified and as the functions of the cells bearing these markers become realized, the diagnostic capabilities of the instrument will increase manyfold. More powerful computers will enhance the analysis and allow increase in the flow rate speed of the instrument.

The applications of the flow cytometer are endless. The instrument has already had a tremendous effect on the fields of science and medicine. In the future, such a machine will no doubt prove to be essential to clinicians and researchers alike.

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Questions Commonly Asked by Students

Question 1: Why is the instrument called a “flow” cytometer?

Flow refers to the method by which cells travel through the machine; cytometry is the measurement of cells.

Question 2: What type of laboratory tests would a physician order that would employ a flow cytometer?

Flow cytometers can be used to diagnose leuke-

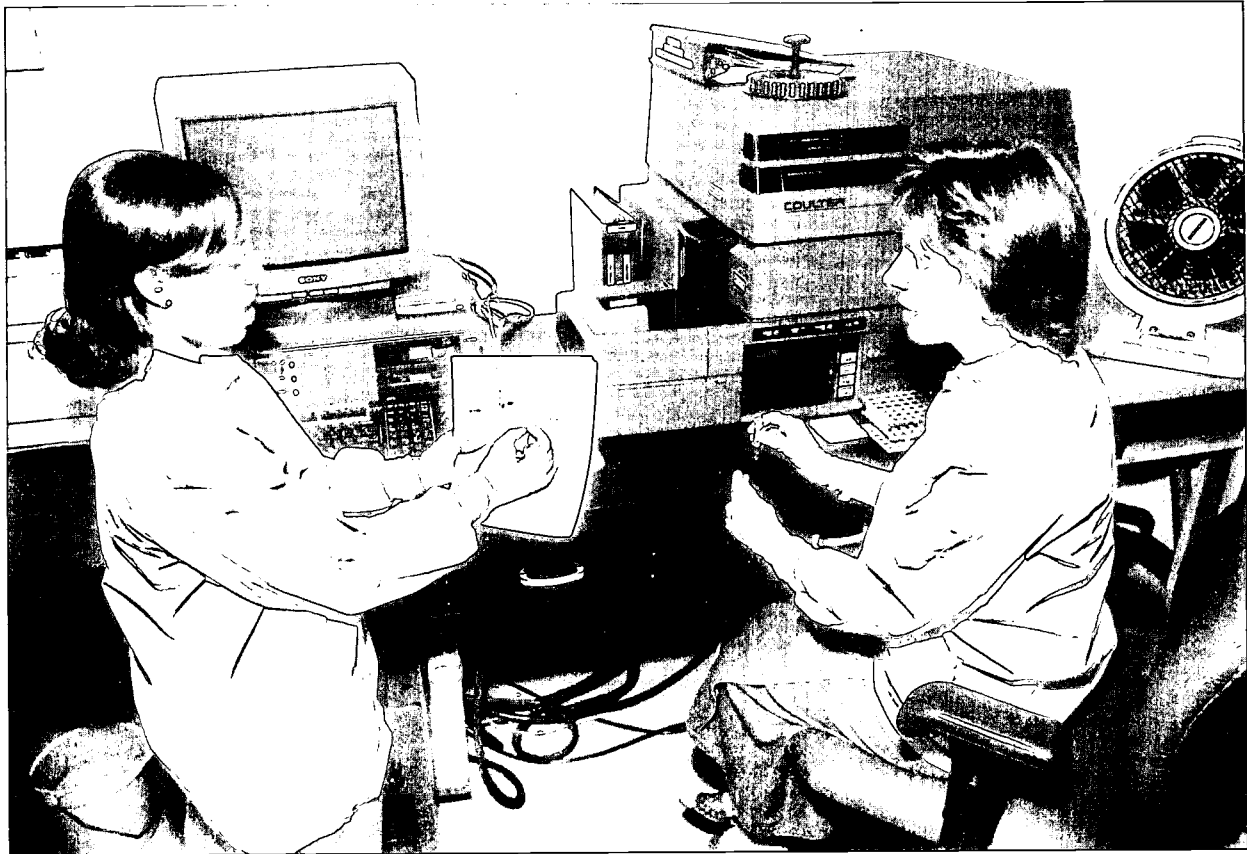
Possible future uses of flow cytometry include:

- 1) identification of DNA in microbes*
- 2) genetic screening*
- 3) studying pathogenesis of disease*
- 4) monitoring effects of therapy and treatment.*

mias and to monitor and provide prognostic information on patients with cancer. The flow cytometer is invaluable for identifying patients with Acquired Immune Deficiency Syndrome (AIDS).

Question 3: How much does a flow cytometer cost?

Most common flow cytometers cost in the range of \$150,000 to \$350,000 at this writing. The more expensive models are usually found in medical and industrial research laboratories.



Bridget L. Langley instructs Roxie Bell, a senior medical technology student at LSU Medical Center, School of Allied Health Professions, in the use of flow cytometry.

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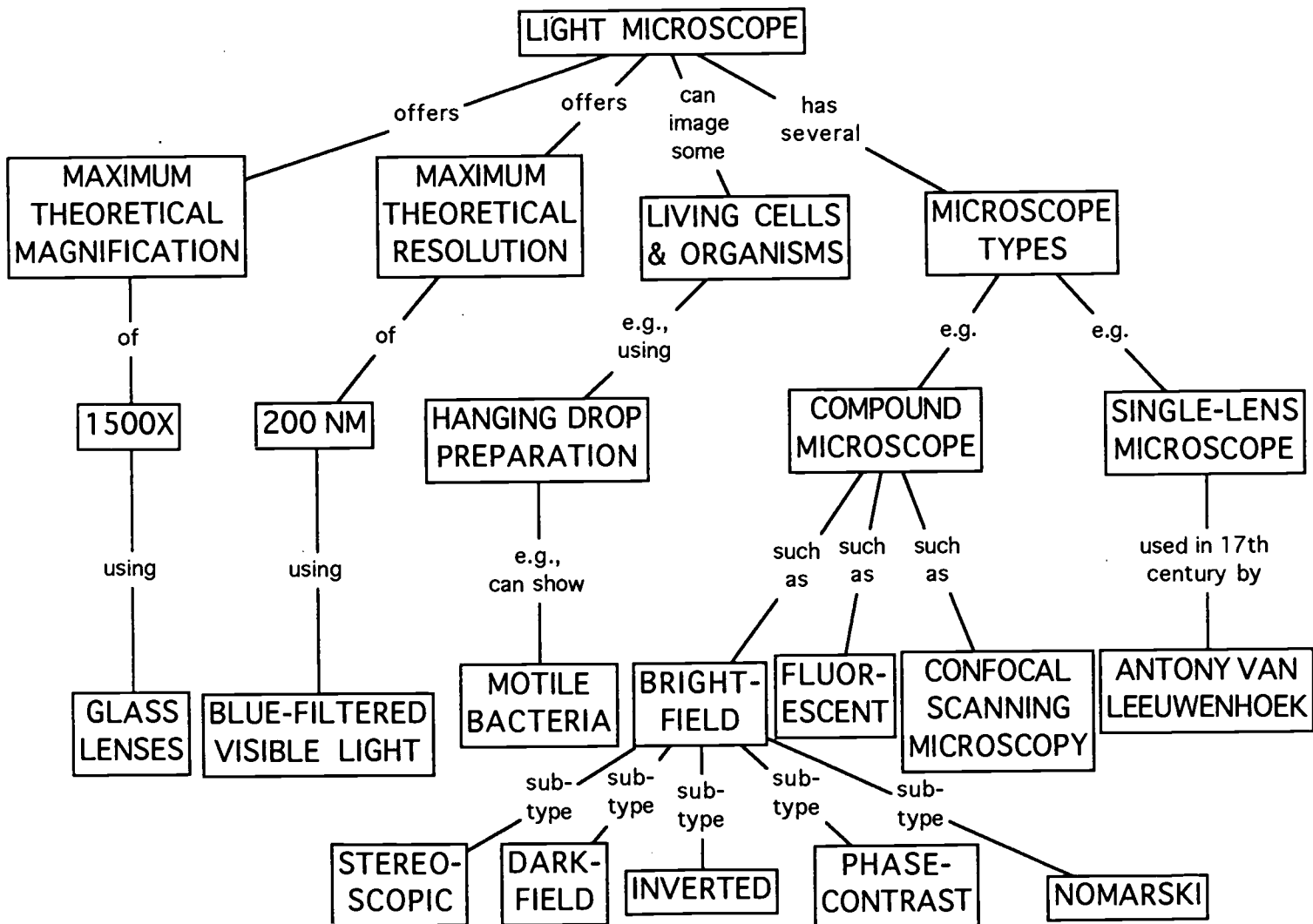
CENTRIFUGATION OF BIOMOLECULES

5

Martha M. Juban and Mary D. Barkley

Overview

1. Introduction to centrifugation
2. Types of centrifuges
3. Separation of methods
4. History of the analytical ultracentrifuge



Centrifugation of Biomolecules

Consider a jar of muddy water scooped from a river. Though the mixture is too cloudy for its components to be identified, we would expect it to clear if the jar is left undisturbed. We might see a layer of large pebbles accumulate very quickly on the bottom of the jar. Smaller particles will fall on top of the larger ones because they take longer to settle out. Silt will layer on top of the pebbles, until eventually the water above will be clear. The force of gravity has achieved the separation, based on the fact that particles of different mass sink at different rates.

Centrifuges are instruments that separate macromolecules.

Centrifuges overcome diffusion and gravity to separate macromolecules.

Two primary types of centrifuges:

- 1) preparative
- 2) analytical.

Homogenate refers to being uniform in emulsions, suspension, or nature.

Centrifuges are laboratory instruments used to achieve separations for which gravity alone would be too weak or too slow. Large molecules, such as proteins, will *not* settle out at 1 g (the earth's gravitational acceleration), regardless of how long we leave the jar undisturbed, because *diffusion* (the random thermal motion of the liquid medium) provides sufficient energy to keep the molecules uniformly suspended. In a centrifuge, mixtures of particles are held in tubes that spin rapidly under centrifugal forces that can be thousands of times as powerful as *g*, overwhelming diffusion and causing sedimentation to occur at faster rates.

Biologists use two primary types of centrifugation, *preparative* and *analytical*. Preparative centrifugation is a purification technique used to separate cells, subcellular organelles such as ribosomes, and macromolecules, such as proteins and DNA, from tissue homogenates. After pure samples have been obtained, analytical techniques can be used to study their sedimentation products in a centrifugal field. Analytical centrifuges include optical systems for observation of the material during sedimentation, allowing calculations of molecular shape and mass and estimates of sample purity.

Sedimentation theory is based on Newton's second law of motion. Consider a sphere of mass m suspended in a solution of density r . Three forces act on the particle (see Figure 5-1).

(1) The *gravitational force* is proportional to the mass of the particle, m , and the acceleration of the gravitational field, g .

$$F_{\text{gravitational}} = mg$$

(2) Archimedes' principle states the *buoyant force* is equal to the weight of the displaced fluid.

$$F_{\text{buoyant}} = m_b g$$

Three forces act upon the particle undergoing centrifugation:

- 1) gravitation
- 2) buoyancy
- 3) frictional drag

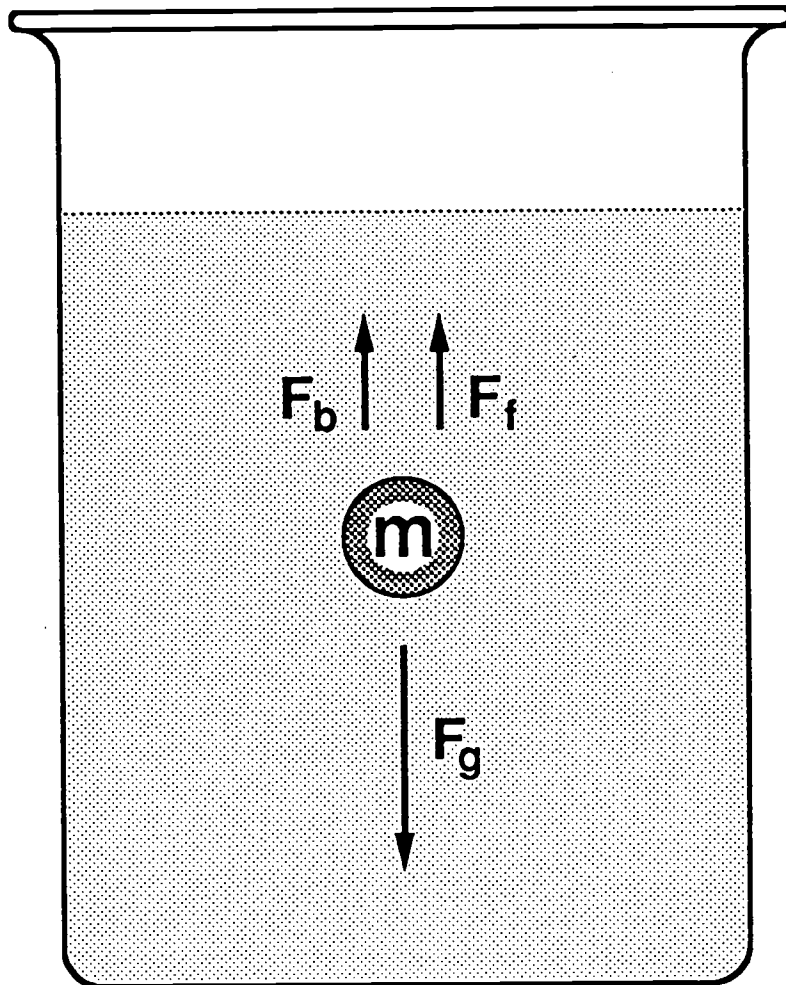


Figure 5-1
Gravitational, buoyant, and frictional forces act on the suspended particle.

where $m_b = m \bar{v} r$, \bar{v} is the partial specific volume, (the volume that each gram of the particle occupies in solution) and r is the solution density (the weight of each mL of solution).

$$F_{\text{buoyant}} = m \bar{v} r g$$

m is mass (not molecular weight) and is expressed in grams.

Initially, the driving force causing the sphere to sink is the difference between the gravitational force and the buoyant force. As the sphere gains speed, it experiences the third force.

(3) The *frictional force* is proportional to its velocity, v , and frictional coefficient, f . The frictional coefficient is determined by the size and shape of the particle; smooth, compact particles have less frictional drag than large, irregularly shaped ones.

$$F_{\text{frictional}} = f v$$

The particle reaches a terminal velocity when the combination of buoyant and frictional forces acting upwards balance the gravitational force acting downwards.

In a centrifugal field, the centrifugal acceleration is $w^2 r$ (instead of g in the gravitational field), where w is the angular velocity of the rotor in radians/second and r is the distance from the center of rotation (see Figure 5-2). The centrifugal force is given by

$$F_c = m w^2 r$$

For a particle suspended in liquid medium in a centrifuge, the particle “sediments” in this field at a constant velocity, v , when the three forces come into balance (see Figure 5-3).

$$\begin{aligned} F_c &= F_b + F_f \\ m w^2 r &= m \bar{v} r w^2 r + f v \end{aligned}$$

The *sedimentation velocity* of the particle is then

$$v = \frac{m(1 - \bar{v} r) w^2 r}{f}$$

This equation tells us:

1. The sedimentation velocity is directly proportional to the strength of the centrifugal field, $w^2 r$.

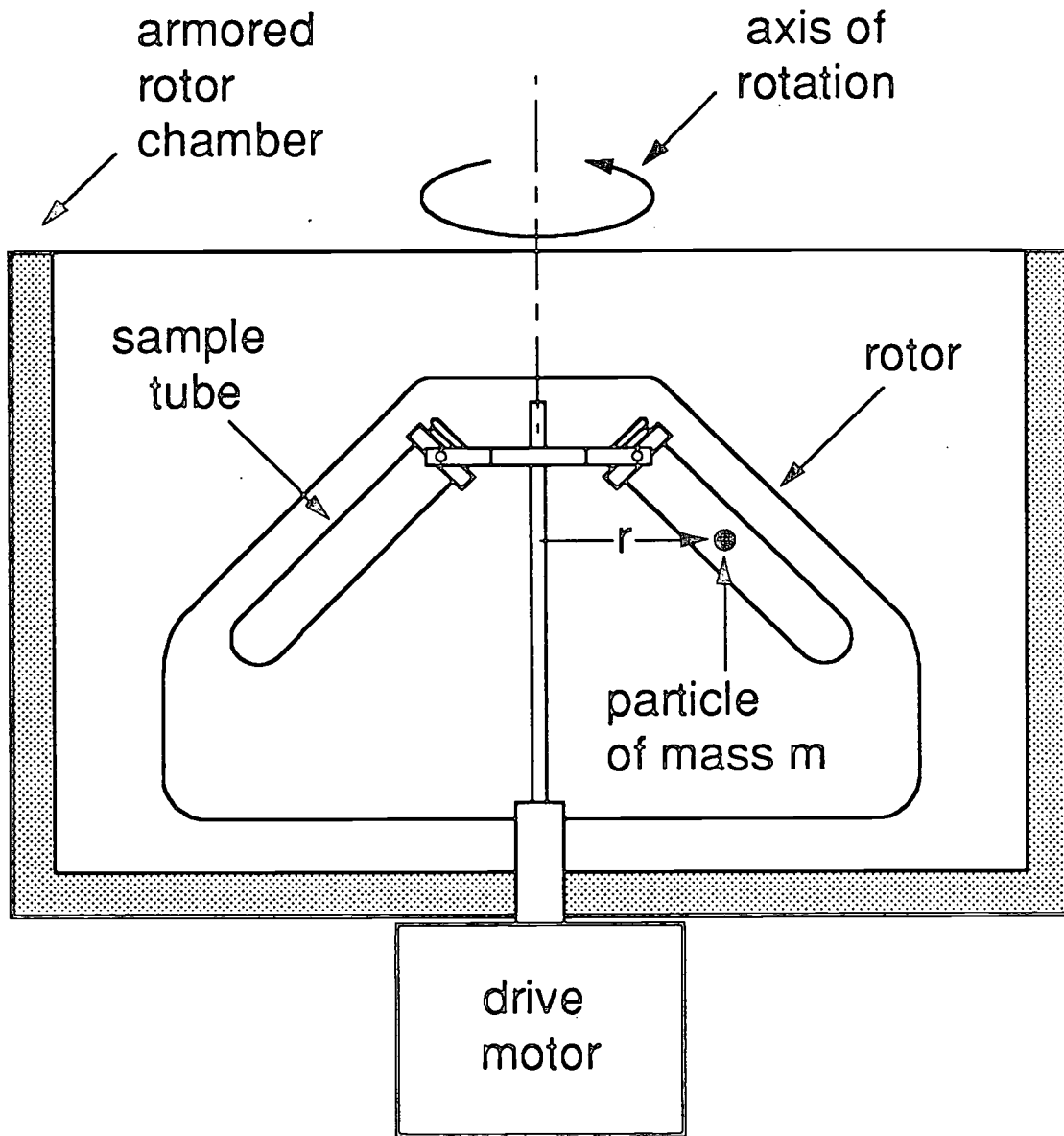


Figure 5-2
Sample tubes in the rotor chamber of a centrifuge.

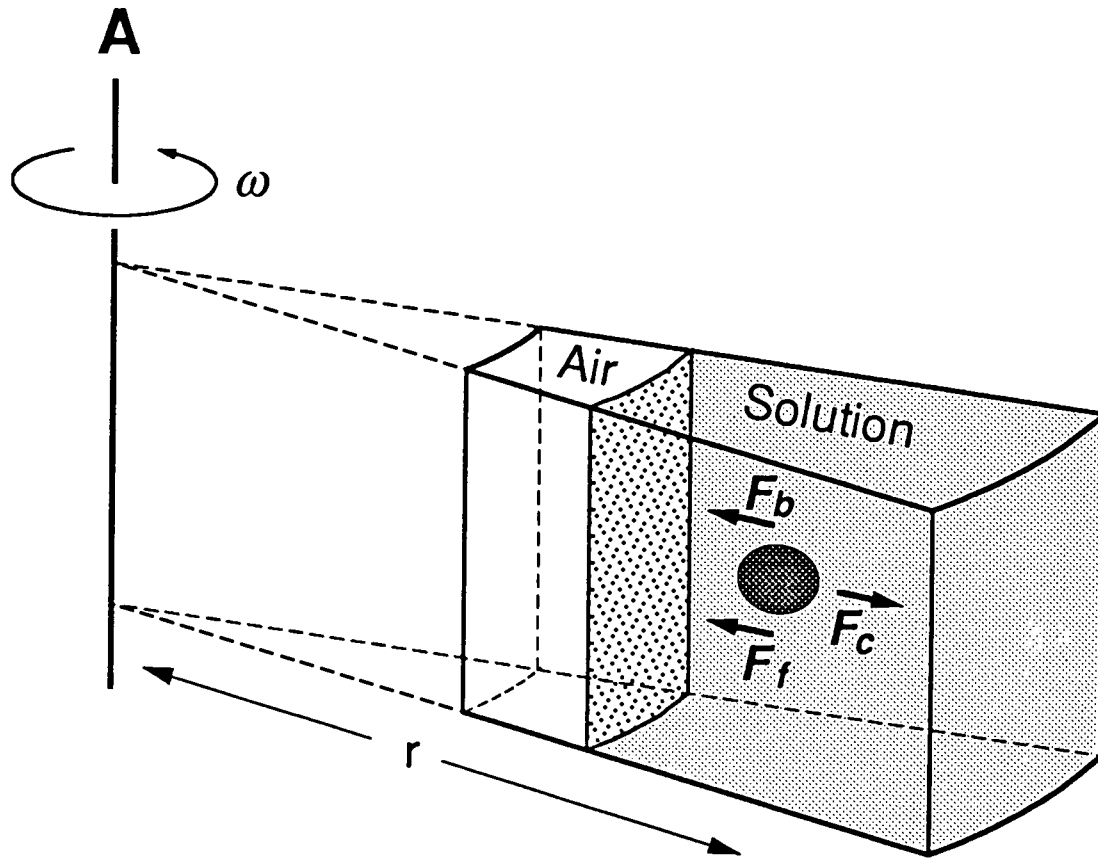


Figure 5-3

The molecule experiences a centrifugal force equal to the product of its mass, its distance from the axis of rotation, and the square of its speed of rotation.

2. The sedimentation velocity of a particle is directly proportional to its mass, m .
3. Denser materials have smaller partial specific volumes, \bar{v} . The buoyancy factor $(1 - \bar{v} \rho)$ means that a denser particle will sediment more rapidly than a less dense particle.
4. Because the shape of the particle affects its frictional coefficient, f , shape also influences the sedimentation velocity. Sedimentation velocity depends on the density of the solution. If $\bar{v} \rho < 1$, particles will sink because they are more dense than the solution, if $\bar{v} \rho > 1$ particles will float to the top of a denser solution, and if $\bar{v} \rho = 1$ the particle will not move.

Sedimentation coefficient s is usually reported in Svedberg (S) units.

It is very useful to characterize particles by a measure of sedimentation that depends on the properties of the particle and solution but is independent of the speed of the rotor. Therefore the *sedimentation coefficient*, s , is defined as the sedimentation velocity per unit of centrifugal force.

$$s = \frac{v}{\omega^2 r} = \frac{m (1 - \bar{v} \rho)}{f}$$

1 Svedberg equals 10^{-13} seconds.

Typical values for s range from 1-100 Svedbergs.

Sedimentation coefficients are expressed in *Svedberg units*, S . A Svedberg is equal to 10^{-13} second. The unit is named for Professor Theodor Svedberg, a Nobel Prize winner in 1926 for his pioneering work in centrifugation. A well-known example of characterizing particles by their sedimentation coefficients is the ribosome: an intact *E. coli* ribosome is called a 70 S particle, which can be dissociated into 30 S and 50 S subunits.

Distance of particles from center of rotation is in centimeters, whereas the speed of rotation is given in revolutions per minute.

The centrifugal field generated by a centrifuge is often expressed in terms relative to the earth's gravitational field as $1g = 981 \text{ cm/second}^2$. When the distance of particles from the center of rotation, r , is given in centimeters and the speed of rotation is given in revolutions/minute, the relative centrifugal field, RCF, is given by:

$$\text{RCF} = 11.18 r (\text{rpm}/1,000)^2$$

This *number times g* is the ratio of the weight of the particle in the centrifugal field to the weight of the particle when acted on by gravity alone.

Types of Centrifuges

Centrifuges are classified according to size and speed, generally into three types:

Centrifuges are classified according to size and speed and can be:

- 1) *low speed*
- 2) *high speed*
- 3) *ultracentrifuge.*

1. *Low speed* centrifuges capable of 2,000-6,000 rpm are used to collect samples that sediment rapidly, such as whole cells and cellular debris. Small bench-top models hold tubes of 1-2 mL volume and may pellet the sample in 1-2 minutes. Larger capacity centrifuges generate fields up to 8000 g, and are used for purification of larger quantities of material in a refrigerated rotor chamber. Rotors of both fixed-angle and swinging-bucket types are available in a range of sizes from 12 mL tubes to 500 mL bottles.
2. *High speed* refrigerated centrifuges with rotor speeds of 18,000-22,000 rpm can generate centrifugal fields in the range of 60,000 g and are used to collect samples such as nucleic acids and protein precipitates from volumes of 10-50 mL.
3. *Ultracentrifuges* ("ultras") are capable of speeds from 35,000 to 80,000 rpm, generating relative centrifugal fields of 700,000 g in temperature-controlled, evacuated chambers. Evacuating the rotor chamber minimizes frictional resistance between air and the spinning rotor. For safety's sake the rotor chamber is encased in heavy armor plating. Preparative ultras are required for sedimenting viruses and smaller cell components such as ribosomes.

Sedimentation velocity can provide shape characteristics of macromolecules.

Analytical ultras include optical systems that permit observation of the particles during sedimentation. Two different types of experiments are possible, termed *sedimentation velocity* and *sedimentation equilibrium*. In sedimentation velocity experiments, the centrifuge is run at relatively high speeds so that the macromolecules being studied move down the length of the sample cell as a boundary that can be analyzed as a function of time. The sedimentation velocity observed can be used to calculate sedimentation and frictional coefficients for the molecule. Comparison of these values to various theoretical ideal models provides clues to the shape of the molecules themselves.

In sedimentation equilibrium experiments, the centrifuge is run at relatively slower speeds for longer times so that an equi-

librium is established between the processes of sedimentation and diffusion. At equilibrium, the opposing forces acting on the molecules within the sample cell are balanced so that the concentration gradient of the molecules does not change with time. The concentration gradient that forms at equilibrium assumes an exponential shape as more solute distributes down the length of the sample cell (see Figure 5-4). A helpful illustration may be to think of the earth's atmosphere as being in a state of sedimentation-diffusion equilibrium. The sedimenting force, gravity in this case, is not great enough to move all the particles to the bottom of the atmosphere. Diffusion establishes a gradient in which the concentration of particles decreases toward the upper atmosphere.

Data obtained from a centrifuge-induced equilibrium state can be used to calculate the molecular weight of the solute. (A plot of the log of concentration vs. radius^2 for a single solute at equilibrium gives a line whose slope is proportional to the molecular weight). The method can be used for molecular weights that range from several hundreds to several millions. If the molecule undergoes a reversible association-dissociation reaction to form more complex structures, the change in molecular weight is discernible from the sedimentation analysis. Different types of interactions can be studied, including self-association (the binding of small molecules to macromolecules) and heterogeneous macromolecular associations. In addition to the sizes of the molecules, sedimentation equilibrium experiments can determine the stoichiometry of the reaction and its equilibrium constant. The equilibrium constant quantitates the strength of the binding between molecules and can be used to calculate changes in thermodynamic parameters (e.g., free energy and entropy) whose magnitudes indicate the types of interactions involved in the reaction.

Sedimentation equilibrium data allows sizing of molecules.

Separation Methods

Three main techniques are used for centrifugal separations.

1. *Differential centrifugation* is the simplest and most commonly used method for separating particles based on their size and density. Initially, the sample components are uniformly suspended throughout the tube. The sedimentation rate of a particle is proportional to particle mass — more massive particles sediment faster to form a *pellet* at the base of the centrifuge tube. The liquid above the pellet, the *supernatant*, contains smaller

Differential centrifugation separates particles based on size and density.

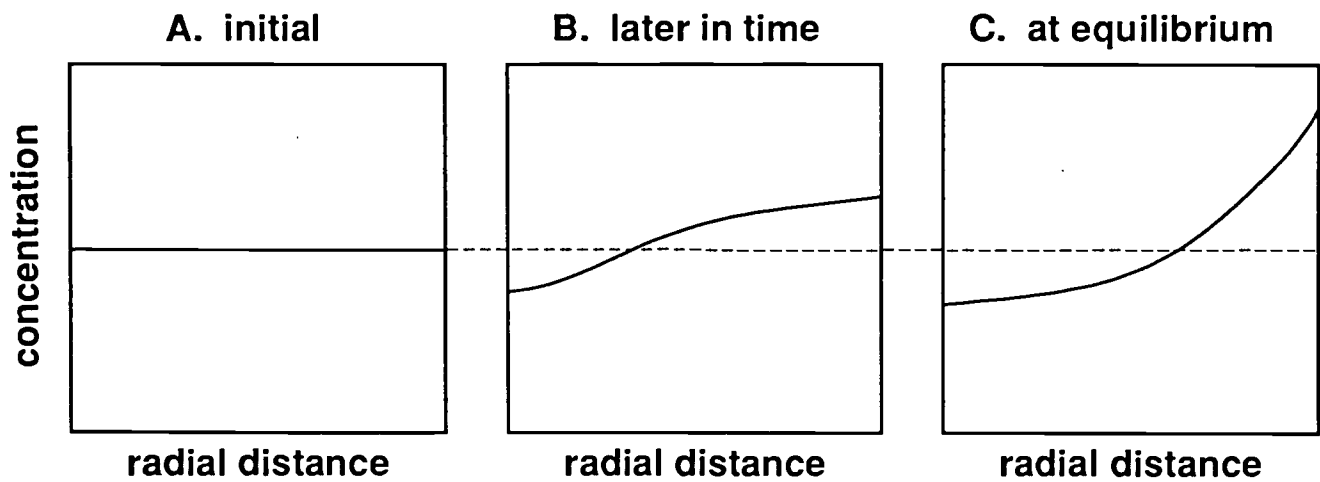


Figure 5-4

A sedimentation equilibrium experiment.

Scan A: solute is uniformly distributed throughout the sample cell.

Scan B: solute is sedimenting toward the bottom of the cell.

Scan C: the concentration gradient at equilibrium.

Later scans would show no change from C.

particles which take longer to sediment. A method for differential centrifugation increases the applied field in a stepwise manner and requires collecting the pellet and pouring off the supernatant in a series of steps that yield fractions enriched in particles of the same sedimentation coefficient. Any particle initially present may be found in the pellet, but purity of the fractions can be improved by repeated washing and resuspension of the pellet.

Differential centrifugation can be used for the isolation of ribosomes from *E. coli*. One procedure uses an SS34 rotor to centrifuge the cell lysate for 15 minutes at 10,000 rpm. The remnants of cell walls and cell membranes will pellet, while most of the ribosomes remain in the supernatant. The supernatant is transferred to an ultracentrifuge tube to spin at 48,000 rpm for 15 hours. The ribosomes pellet along with proteins and other contaminants and must be further purified.

Rate-zonal centrifugation sees the differences in sedimentation rates due to particle size and shape.

2. *Rate-zonal centrifugation* through a density gradient is another separation method that takes advantage of differences in sedimentation rates due to size and shape. Compared to differential centrifugation, separations may be significantly improved with a rate-zonal method, but sample capacity per tube is greatly reduced. The density gradient is a liquid column whose density increases toward the bottom of the centrifuge tube but whose maximum density does not exceed that of the densest particle to be separated.

Discontinuous or step gradients can be formed by overlaying volumes of solutions of decreasing densities. Continuous gradients can be formed by layering solutions of different densities. For example, a 5-20% sucrose gradient can be formed by layering equal volumes of 5%, 10%, 15%, and 20% sucrose in a tube and leaving it to diffuse for 12-18 hours. The sample is carefully layered on top of the pre-formed density gradient. During centrifugation, the particles travel through the gradient to segregate by their sedimentation coefficients into discrete bands. The gradient material stabilizes the bands so the particles do not crash to the bottom of the tube. Timing is critical. Enough time for band formation is essential, but centrifugation must be stopped before the fastest-moving band pellets at the bottom of the tube.

The gradient material must be stable and inert with respect to the sample of interest. DNA and RNA are usually fractionated in solutions of cesium chloride. Sucrose gradients are

generally used for protein and organelle separations though glycerol and commercial preparations such as Ficoll (a high molecular weight polymer of sucrose) are also useful.

Analysis of the distribution of the sample in the gradient may be as simple as visual inspection or may require specific assays for the type of molecule involved. Recovery methods depend on the sample and the type of tube used. If a band is visible in an open tube, a Pasteur pipette placed into it makes recovery simple. If the separation requires a sealed plastic tube, the band may be recovered by inserting a hypodermic syringe through the tube wall. In many cases, it may be necessary to remove the gradient fractions sequentially, either through a hole in the bottom of the tube or by pumping out from the top of the tube, and to analyze each one.

Isopycnic centrifugation is useful in separating particles of similar size but differing densities.

3. *Isopycnic centrifugation* in a density gradient depends only upon the buoyant density of the particle and is, therefore, useful for separating particles of similar size but differing densities. The sample is layered on top of a liquid column whose maximum density must exceed that of the densest particle to be separated. As sedimentation occurs, particles reach an isodensity point in the gradient and will sediment no further. This technique is not time-dependent but is an equilibrium method.

Density gradients are not always pre-formed but may be self-forming during centrifugation, as in the case of using chloride gradients. A commonly used method of separating plasmid DNA from chromosomal DNA and other contaminating cellular components in bacterial lysates uses ethidium bromide and a cesium chloride gradient. Ethidium bromide molecules insert between the base pairs of a DNA double helix. More ethidium bromide intercalates into chromosomal DNA than into plasmid DNA, making the buoyant densities significantly different. During centrifugation at 45,000 rpm over a period of 16 or more hours, the DNA-ethidium bromide complexes migrate to their isodensity points in the tube and appear as distinct bands. The bands are visible in ordinary light and can be removed from the tube with a syringe. The ethidium bromide can be extracted with organic solvents, resulting in a very pure plasmid DNA solution for experimentation (see Figure 5-5).

A laboratory technician faced with a separation problem generally begins with a fractionation procedure published by someone else engaged in similar work. It is important to note that a procedure may not achieve the same separation when used with different equipment. The size and shape of the centri-

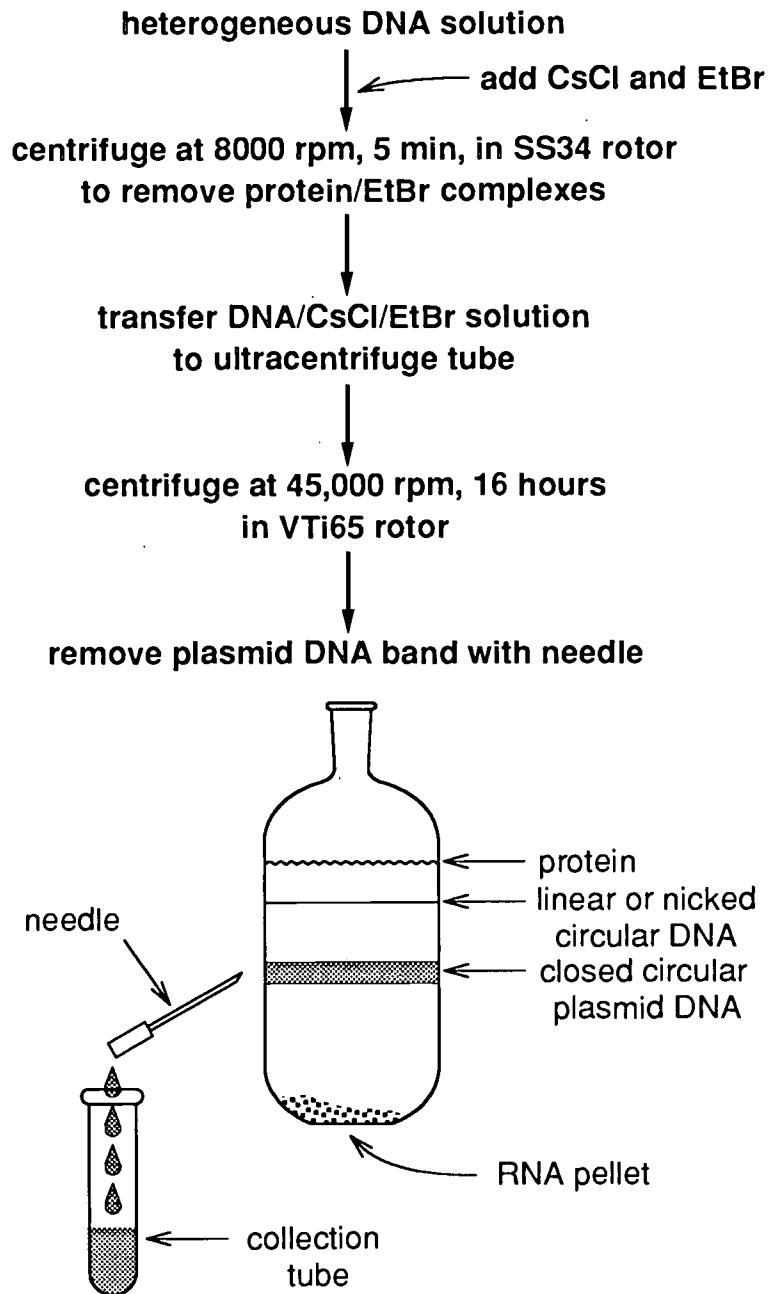


Figure 5-5
An isopycnic method for purification of plasmid DNA.

84 Centrifugation of Biomolecules

Size and shape of centrifuge tubes and the configuration of the rotor are important parameters to consider with separation procedures.

fuge tubes and the configuration of the rotor are important parameters to consider. For example, the DNA purification outlined requires 16 hours at 45,000 rpm in a VTi65 rotor but 48 hours at the same speed in a Ti50 rotor.

A History of the Analytical Ultracentrifuge

The first ultracentrifuge was introduced by Svedberg in 1923.

Modern molecular biology owes much to the groundbreaking work done in the first two decades of the 20th century by Theodor Svedberg, a Swedish professor of physical chemistry working in what was termed *colloid chemistry*. Macromolecules were not believed to exist; most researchers assumed that ordinary small molecules clustered together in particles large enough to scatter light but of undefined mass. Svedberg was interested in studying the size and distribution of size of colloidal particles. While a visiting professor at the University of Wisconsin in 1923, he constructed the first centrifuge incorporating an optical system so that observation of the sedimentation was possible during rotation. He returned to the University of Uppsala in Sweden and, in 1924, built an improved machine to look at gold sols using parts from a cream separator. The instrument was named an *ultracentrifuge*, though its maximum speed was about 10,000 rpm, or approximately 5000 g. Protein studies began with ovalbumin and horse hemoglobin, and the hemoglobin results were quite unexpected. Rather than finding a range of sizes of colloid particles, the experiments indicated a "monodisperse colloid;" that is, the individual particles were all the same size. Subsequent experiments with hemocyanin again produced surprising results. The protein was much larger than expected, and was also monodisperse.

Svedberg was the first to present evidence that proteins were composed of large molecules or colloidal aggregates.

These observations led to extensive protein studies and continued improvements in instrumentation. The first high-speed instrument was built in 1925, furnishing centrifugal fields up to 100,000 g at speeds of 45,000 rpm. Improvements in stability, temperature control, drive mechanisms, and the sample-containing cells continued at a rapid pace. Reducing the size of the rotor made possible fields up to 600,000 g in 1933, but such rotors exploded after only a few runs.

The optical systems used both absorption and refraction of light of a certain wavelength from a mercury arc lamp to determine the concentration distribution in the rotating sample. In the absorption method first used by Svedberg, the image of the rotating liquid column was focused on a photographic plate.

In order to determine the concentration at each point in the cell, the degree of darkening of the plate was compared to a "concentration scale" made by running a series of known dilutions of the solution.

Despite its difficulties and complexities, in the 1930s, the ultracentrifuge became the tool of choice for determining the molecular weights of proteins. The fact that soluble proteins consist either of a single (monodisperse) or of a few (paucidisperse) molecular species of definite mass became apparent. The theoretical development of sedimentation equilibrium for molecular weight calculations and sedimentation velocity for sedimentation coefficient values paralleled the evolution of the instrument.

During the 1930s, ultracentrifuges became a tool for determining molecular weights of proteins.

Svedberg and others studied respiratory proteins from the animal kingdom, finding identical sedimentation coefficients for proteins from different species belonging to the same family but differing values for those from a different group. Though Svedberg himself visualized proteins as colloidal aggregates, his data were the earliest evidence that proteins are composed of subunits.

The TMV was sized and its shape characterized from sedimentation coefficients before it was visualized with electron microscopy.

Important work was also being conducted in the U.S. on plant virus proteins. The size and shape of the tobacco mosaic virus was successfully predicted from the sedimentation coefficient before the rod-like virus particles were ever seen with the electron microscope.

In 1940, Theodor Svedberg and Kai Pederson published their classic reference work, *The Ultracentrifuge*, including detailed accounts of design, theory, and experimental methods, as well as tables of data of molecular constants for many proteins. The field exploded after the first commercially available analytical ultracentrifuges were put on the market in 1947, the Spinco Model E.

In the 1950s, many researchers were concerned that nothing was left to accomplish with the ultracentrifuge other than making measurements on yet another protein. However, advances in thermodynamic theory and in nucleic acid research led to new applications in characterizing peptide, protein, and nucleic acid molecules and their interactions. In 1958, Matthew Meselson and Franklin Stahl used data from the analytical ultracentrifuge as direct evidence for the semi-conservative replication of DNA that had been hypothesized by Watson and Crick in 1953. Meselson and Stahl grew *E. coli* labeled with a heavy isotope of nitrogen by growing bacterium in media containing N^{15} . Sedi-

Experiences with analytical ultracentrifugation in the late 1950s supported the Watson-Crick DNA replication hypothesis.

mentation equilibrium experiments showed the buoyant density of the bacterial DNA varied directly with the fraction of N^{15} label it contained. The labeled culture was transferred to normal N^{14} media, and after one generation time had elapsed, Meselson's data showed an accumulation of half-labeled DNA molecules while the fully-labeled DNA was depleted. The results supported the Watson-Crick hypothesis that DNA molecules duplicate by separating the double chains and using each chain as a template for the synthesis of its complement.

During the 1970s, the computer was linked to the ultracentrifuge, eliminating much of the tedious work involved in data acquisition and analysis, but the 1980s saw a decline in the use of ultracentrifugation. Biologists and biochemists used simpler but less accurate techniques, like gel electrophoresis and gel chromatography, to estimate molecular mass. The venerable Model E was no longer commercially available, and the number of instruments in working order diminished. However, in 1991, the Beckman company made available a greatly improved model, the X-LA, which has opened the door for a new generation of researchers in the field (Figure 5-6).

Biochemists, biophysicists, and molecular biologists still have much to learn about the relationship between the structure and the function of protein and nucleic acid molecules, and their interactions. Obtaining sufficient quantities of pure material to study is usually the first problem that researchers must solve, but technical advances like the automated synthesis of peptides, the expression of proteins by recombinant DNA systems, and site-directed mutagenesis are making interesting molecules more available than ever. The rigorous, quantitative studies made possible by the analytical ultracentrifuge are leading to greater understanding of the structure and function of biological macromolecules.

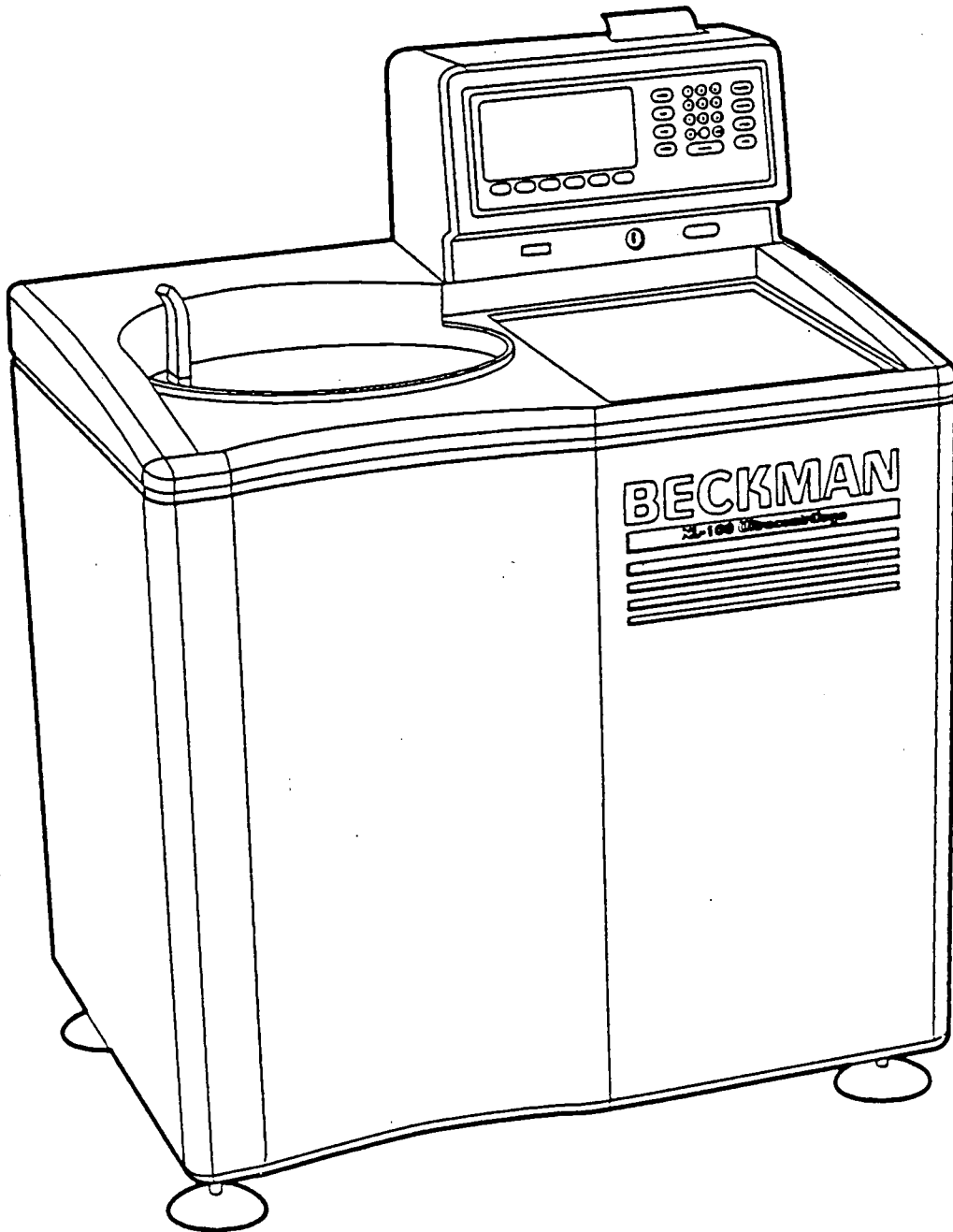


Figure 5-6
Schematic of the Beckman Optima XL 100k
(Used with permission of Beckman Instruments, Inc.)

Questions Most Commonly Asked by Students

Question 1: How do you determine how long a sample should be centrifuged?

Compare whatever is known about the sample to our own experience in using the equipment available. Often we modify a basic procedure, taking into account the parameters in the equation for sedimentation velocity. For example, we assume more time would be needed for samples with smaller sedimentation coefficients, for separations in more dense media, or for slower rotor speeds.

Question 2: What is the best way to measure the molecular weight of a protein?

Many methods are used to measure molecular weights, and each has advantages and disadvantages. Estimates of molecular weight can be determined from gel electrophoresis and gel chromatography— techniques where the rate of the unknown sample in moving through a gel is compared to the speed of known standards. The easiest way to get accurate values for a single molecule is to use a mass spectrometer. If we need to measure the size of an aggregate of associating molecules as they exist in solution, the analytical ultracentrifuge is the method of choice.

Question 3: How does the optical system of the analytical ultracentrifuge “see” into the sample cell?

The sample solution is placed in a housing between two quartz windows. The rotor has a hole over the window so that light can reach the sample. The optical system can measure how much incident light is absorbed at each radial position down the length of the sample cell.

Question 4: Why does the optical system measure absorbance to determine the concentration gradient?

The amount of light of a specified wavelength absorbed by a sample is proportional to the number of absorbing molecules. Absorbance and con-

centration are related by the Beer-Lambert Law ($A = \epsilon c d$, where c is the molar concentration of solute, d is the distance the light travels through the sample, and ϵ is a proportionality constant called the extinction coefficient which is characteristic for each type of molecule.) Not all analytical ultracentrifuges use absorption optic. Some have systems that use refraction of light to measure concentration.



Professor Mary D. Barkley is seated at the computer which controls LSU's analytical ultracentrifuge. Dr. Barkley's graduate student, Maryam Javadpour, is loading the rotor into the instrument.

References and Suggested Reading

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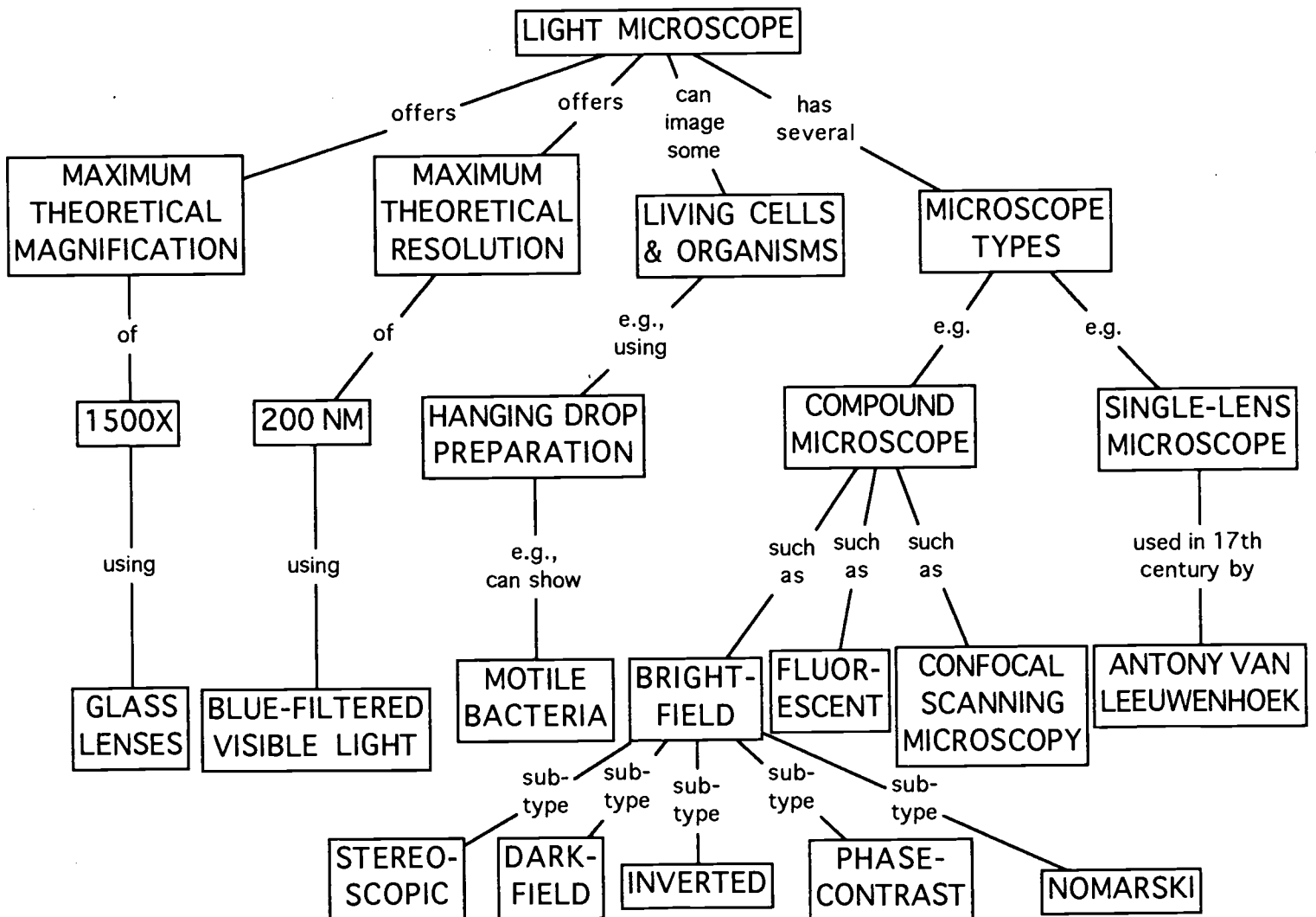
ADVANCEMENTS IN LIGHT MICROSCOPY

6

Elaine Cox

Overview

1. Basics of light microscopy
2. Bright-field microscopy
3. Steps in using a compound microscope
4. Variations in bright-field microscopy
5. Different types of compound microscopes



Advances in Light Microscopy

Introduction

The most common microscopes found in most high school and college science labs are the bright-field and stereoscopic microscopes.

The use of light microscopes is fundamental to the study of life — especially tissues, small plants, and animals and their parts, as well as microorganisms (bacteria, yeasts, molds, algae, and protozoans). Viruses and most cell organelles are too small to be seen by light microscopy and must be viewed by electron microscopes (see Chapter 7). The most commonly used microscopes in high school and college laboratories are the bright-field microscope and the stereoscopic or dissecting microscope. Other microscopes useful for specific applications include the phase-contrast microscope, dark-field microscope, fluorescent microscope, inverted microscope, and the more recently developed Nomarski differential interference microscope, confocal scanning microscope, and IC inspection/metallurgical compound microscope.

Hooke used a compound microscope, and Leeuwenhoek used a single-lens microscope.

Microscopy as a science began in the 17th century when, independently, an Englishman, Robert Hooke, and a Dutch linen merchant, Antony van Leeuwenhoek, designed and used simple microscopes to observe living and nonliving materials. Gradually microscopes evolved into a more complex and optically refined instrument, the compound microscope, that has been in use since the mid-1800s. Our modern laboratory microscopes are very similar in basic design and function to those of 150 years ago.

Basic Microscopy

The lens system of a compound bright-field microscope includes the oculars, objective lenses, and condenser.

The parts of a typical compound microscope are seen in Figure 6-1. Compound microscopes have two magnifying lens systems, the oculars (eyepieces) and the objectives, as well as a third lens, the condenser. The most common ocular magnification is 10X, although some oculars magnify 15X. Monocular microscopes have one ocular while binocular microscopes have two. For binocular microscopy, the distance between the oculars must be adjusted to fit the distance between the observer's eyes (interpupillary distance) comfortably. This is accomplished by moving the eyepiece plates in or out or by rotating a thumb-wheel located between the oculars until only one image is seen. Most binocular microscopes are designed to adjust for the difference in the ability of each eye to bend light rays. After using the right eye only to fine focus the right ocular on a prepared

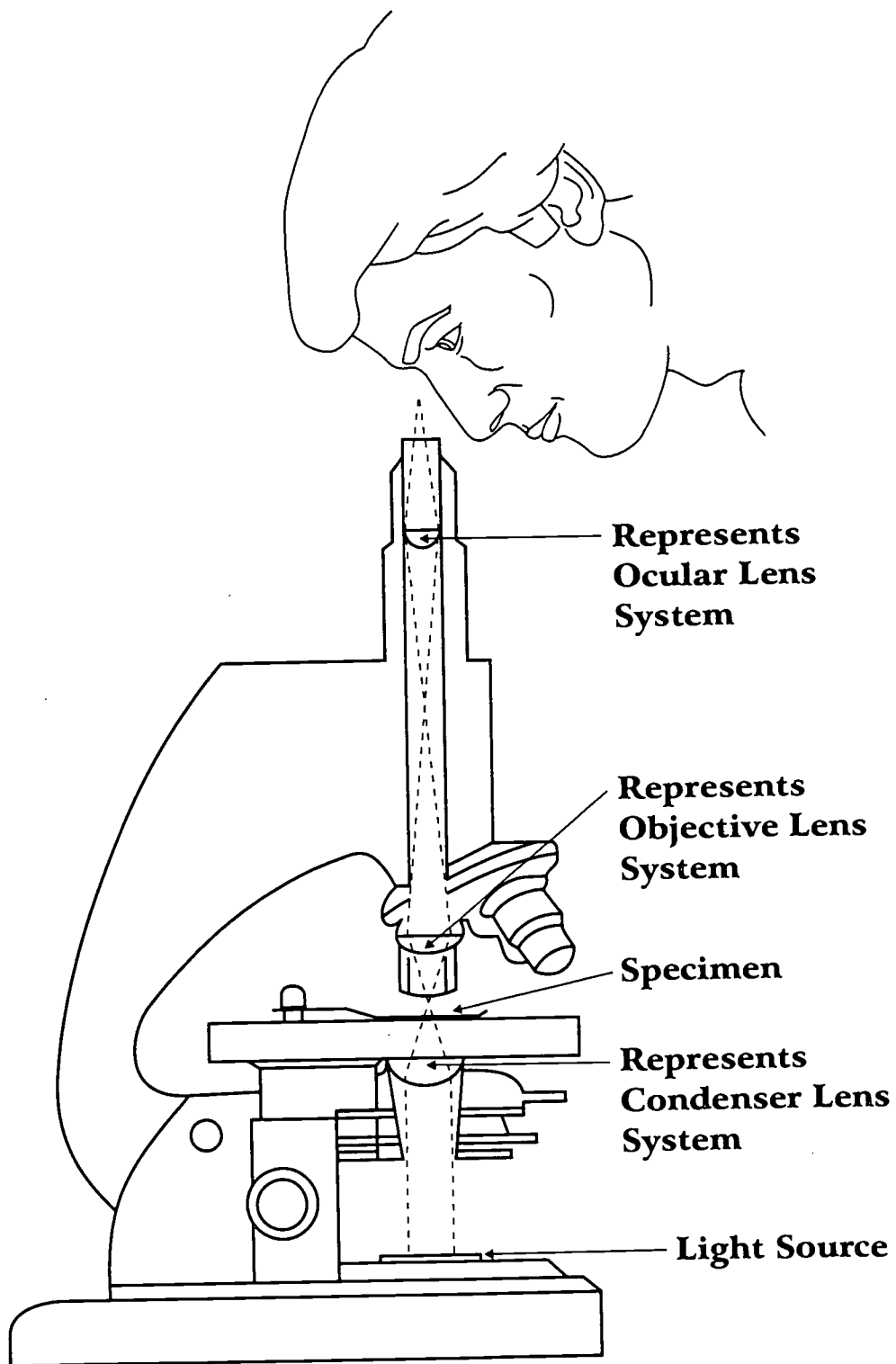


Figure 6-1
Typical parts of a compound microscope.

slide, one should look into the left ocular and focus the image by turning the diopter ring.

Huygenian oculars are relatively simple eyepieces invented by the Dutch astronomer Christian Huygens. The Huygenian ocular is satisfactory, but a modern wide-field ocular gives approximately a 25% larger field of view. In addition, the wide field ocular is better for observers who wear eyeglasses.

The objective lens concentrates the light from the specimen to create an image for magnification.

The ocular, or eyepiece, is the lens system that magnifies the image formed by the objective lens.

The objective lenses typically found on a microscope turret are a low power lens, a high-dry power lens, and an oil immersion lens. Each of these is really a lens system made of several lenses.

The objective lenses gather light coming from all parts of the specimen, form an image, and magnify that image. Two images are produced by compound microscopes. The real image is produced by the objective lens; this image is further magnified by the ocular lens to produce an inverted (upside down) image, the virtual image. Most compound microscopes have at least two objectives: the low power (magnification of 10X) and the high-dry power (magnification of 40X, 43X, or 45X). The oil immersion lens (magnification of 100X) is necessary for observation of most microorganisms, especially bacteria. Another common objective is the scanning objective (magnification of 4X) which is useful for viewing large specimens, such as tissue slides of spinal cord, skin, and motor neurons and whole mounts of small animals such as flukes, *Amphioxus*, and small jellyfish and their larvae.

The types of modern microscope objective lenses that correct optical problems are achromatic, plan achromatic, and apochromatic lenses.

Modern microscope objectives are commonly of the achromatic, plan achromatic, or apochromatic type. Convex lens surfaces produce both spherical and chromatic aberrations. Spherical aberrations create a curved, rather than a flat, image; chromatic aberrations create blurry, rainbow-like images. Spherical aberrations occur because light rays passing through the outer edges and the center of a convex lens do not focus at the same point. These aberrations can be corrected by the use of compensating lenses containing two types of glass (flint and crown) of different shapes and compositions. Achromatic lenses improve both chromatic and spherical aberrations, while plan achromatic lenses are even better. Apochromatic lenses show no chromatic aberration and correct for the curvature of the field so that all objects in the field of view are simultaneously in focus. This type lens is best for photomicrography. Compensating lenses are also used in oculars to correct for chromatic aberration. Microscopes for fine research work may have sophisticated fluorite objectives or objectives specific to the application (e.g., Nomarski or dark-field objectives).

The numerical aperture (NA) of a lens can be thought of as an index of the efficiency of the lens in bending light.

Total magnification is the product of the ocular magnification times the objective lens magnification.

The working distance is defined as the distance from the bottom of the objective lens to the specimen being studied.

Parfocal refers to the specimen remaining in focus when you switch from one objective to another.

The condenser must be positioned correctly to focus and evenly distribute the light shining on the specimen.

The NA of the condenser should be equal to the NA of the objective lens being used.

Each objective has both its magnification and numerical aperture (NA) imprinted on it. Total magnification achievable with an objective is calculated by multiplying the magnification of the objective being used by the ocular magnification (e.g., a low power objective (10X) paired with a 10X ocular gives a total magnification of 100X). Good light microscopes magnify a total of 1000X; better light microscopes can magnify up to 1500X.

The greater the magnification of the objective, the shorter the working distance becomes; in the case of the oil immersion objective, only a thin film of immersion oil separates the lens from the slide. Immersion oil must be used with the oil immersion objective. If light from the condenser hits molecules of air before entering the oil immersion objective, a distorted image results. Immersion oil has essentially the same refractive index as glass (1.515) and its use increases the resolution obtainable with this objective.

Microscope objectives are designed either to be used with or without coverslips. Those objectives to be used without coverslips have the designation "/0" after the tube length; objectives to be used with coverslips are designated "/0.17" after the tube length. Coverslips to be used with the so-designated objectives should be of No. 1½° thickness, 0.17 mm to 0.19 mm.

Parfocal microscopes offer definite advantages to the user. When a specimen is in focus on a low power, it will be in focus or nearly so, when one switches to a higher objective. After changing to a higher power objective, the fine focus knob is used to bring the focus to a maximum. Because the higher power objective "sees" a smaller portion of the specimen, an increase in light is often necessitated. The specimen may also need to be re-centered in the field of view.

The condenser, a special lens found under the stage, focuses the light rays to a single point on the specimen. The numerical aperture of the condenser must be greater or equal to the numerical aperture of the objective in order to use the full capacity of the objective. The Abbe condenser, the most commonly used type, is preferable for all bright-field microscopy applications because its numerical aperture (1.25) is that of good oil immersion objectives. Lesser condensers with lower numerical apertures give poorer resolution. Other condensers used in microscopy are of the achromatic-aplanatic, dark-field, phase-contrast, and Nomarski differential interference types.

For efficient viewing, a mechanical stage is necessary when using an oil immersion objective lens.

The iris diaphragm controls the amount of light passing through the specimen being studied.

The iris diaphragm employs an adjustable aperture that allows adjustment of the light intensity seen by the microscopist.

Regulation of light affects the resolution of the condenser (i.e., decreasing the iris aperture decreases the resolution).

The coarse focus knob is used on lower power magnification while the fine focus knob is used to sharpen the image even further for more detailed viewing.

To prevent eye strain, keep both eyes open, even when using a monocular microscope.

Resolution refers to how small and close individual objects or dots can be and still be recognizable as separate entities.

Resolution depends upon:

- 1) the wavelength of the light used for illumination.*
- 2) NA of the lens system.*

Specimens are placed upon a platform called a stage. Slides are held on the stage by clips and moved by hand. An improvement upon this design is the mechanical stage, in which the slide is held by clips that are moved in two directions at right angles to each other via a gear system by knobs located at one side of the stage. A mechanical stage is necessary for positioning specimens viewed with the oil immersion objective, when even small movement of the slide is multiplied a thousand-fold.

Older microscopes found in high school laboratories rely on a mirror attached to the base of the microscope and an external lamp to provide illumination. The mirror and lamp should be positioned so that maximum light enters the condenser. A light source built into the base of the microscope is the best way to illuminate an object. Tungsten or tungsten-halogen lamps provide good illumination and longer life. A variable voltage regulator offers a greater range of illumination possibilities to the user. An adjustable iris or disc diaphragm also controls the amount of light hitting the specimen. When viewing wet-mount slides, the iris diaphragm should be used to adjust the amount of light hitting the specimen such that resolution is maximized. Viewing stained specimens requires maximum light from the condenser (iris diaphragm open). If more light is needed, voltage (if adjustable) to the lamp should be increased.

The use of both coarse and fine adjustment knobs allows for sharp imaging. The coarse focus knob is used on scanning or low power magnifications and is not used on a parfocal microscope at higher magnifications. The use of the coarse focus knob at high magnification with a non-parfocal instrument must be done with care, as the objective can quickly be rammed into the slide and both can be damaged. The fine focus knob is used to bring the image into sharp focus.

Magnification is an important aspect of microscopy, but resolution is of much greater importance. Resolution is the ability to distinguish two closely spaced objects as two objects. Magnification beyond the limit of resolution is useless since it enlarges blurry images. The limit of resolution of a microscope depends upon two factors: (1) the wavelength of the energy source (light rays for the light microscope) being used and (2) the numerical aperture of the lens systems. The shortest wavelengths give the greatest resolution, so most light microscopes have a blue filter under the condenser to limit the entrance of longer red wavelengths of the light spectrum to the specimen. The numerical aperture describes the efficiency of the lens in bending light rays.

The shorter the wavelength of light used, the greater the resolution.

The higher the NA of the lens, the greater the resolution.

The higher the numerical aperture, the better the resolution of that lens. Using a lens with the highest numerical aperture and the shortest wavelengths of light, the limit of resolution of the light microscope has been calculated to be 0.2 micrometers or 200 nanometers. This means that objects spaced 0.2 micrometers apart can be seen as separate objects, but objects closer together are viewed as one. Only with the most refined optics and newest technologies can we even approach this theoretical limit of resolution.

Improvements in light microscopy during the last century include the development of novel methods to improve resolution through improving the contrast of the specimen. This goal has been accomplished in three ways: the application of stains to the specimen, variations of the bright-field microscope, and the use of computer-aided video imaging.

Bright-Field Microscopy

Compound Bright-Field Microscope

The most commonly used microscope in secondary and college biology laboratories is the compound bright-field microscope. Visible light is transmitted through a specimen that appears relatively dark on a bright background. Specimens to be viewed with the bright-field microscope are positioned on a glass slide. Large protozoans (such as *Paramecium*, *Amoeba*, and *Stentor*) and colorful algae (such as *Volvox*, diatoms, *Spirogyra*, and *Euglena*) are easily observed in the living state on low and high-dry magnifications. Permanent slides can be viewed at all magnifications.

Preparation of the specimen for microscopy depends upon the type of specimen and the type of observations you want to make about the specimen. Wet mounts are easy to make and view if the specimen is carefully selected. A transparent (or nearly transparent) specimen will allow light rays to enter. Thick, opaque specimens are not acceptable for microscopy.

Wet mounts are also useful to view fairly large, living organisms such as protozoans, yeasts, and fungi, or to note reactions of the living cell or organism brought about by changes in the micro-environment on the slide (e.g., addition of chemical attractants, or hypertonic/hypotonic solutions). Wet mounts are easily prepared by adding a drop (containing the specimen already suspended in a liquid) to a glass slide.

Wet mounts are ideal for viewing many living organisms.

Care must be taken to keep air bubbles from forming under the coverslip when it is applied to the slide.

A coverslip is added carefully to the specimen by first dragging one edge of the coverslip in the liquid to flatten out the drop, then gently laying down the coverslip. This procedure reduces the number of air bubbles trapped under the coverslip. If liquid is oozing from under the coverslip, the extra liquid may be blotted by placing a very small piece of paper towel or bibulous paper next to the edge of the coverslip. Too much liquid in a wet mount preparation causes the specimens to respond to pressure of the objectives and to "swim away in currents." (CAUTION: *It is best not to allow students to blot slides of bacteria.*)

A simple stain can be added to the previously prepared and observed wet mount. To do so, remove the slide from the microscope, add a small drop of the stain to one edge of the coverslip, and blot extra liquid away from the opposite corner of the coverslip. This procedure works best if the wet mount has not begun to dry out.

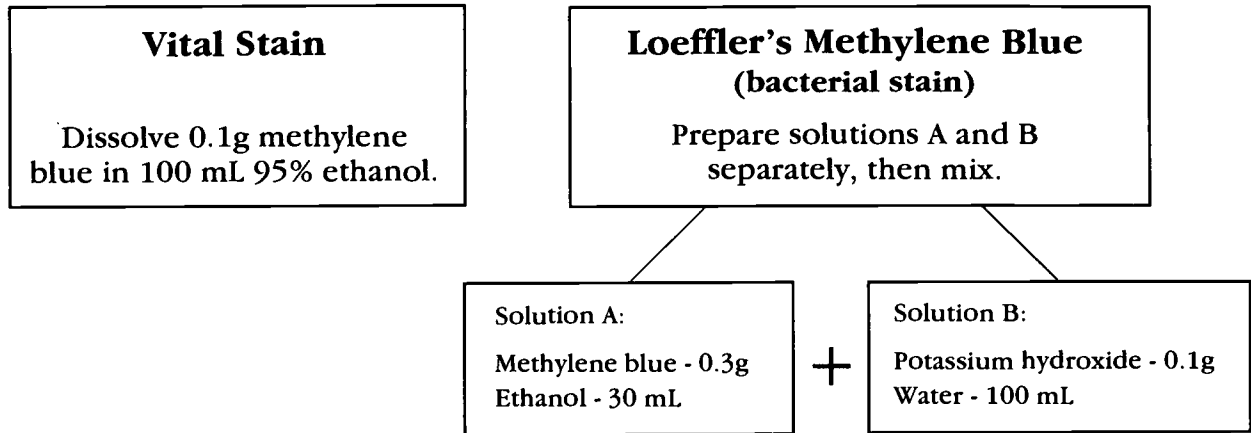
The hanging drop preparation of a wet mount is useful for viewing the motility of small organisms.

The hanging drop preparation is a modification of the wet mount and is useful in observing motility of very small organisms such as bacteria. Coverslips, depression slides, and petroleum jelly are needed. The coverslip is prepared by applying a thin line of petroleum jelly (using the tip of an applicator stick or toothpick) to all four edges on one side of the coverslip. A small drop of the specimen is put in the center of the coverslip. The depression slide is inverted over the coverslip so that the recessed area is placed over the drop of the specimen and gently pushed down. The jelly causes the slide and coverslip to stick together, and when the slide is picked up and inverted, the drop of specimen hangs in the recessed area of the slide. In this environment, protected from drying and air movements, true motility of cells can be more easily distinguished from water current and Brownian movement.

The addition of dyes to stain transparent specimens, such as onion slices, bacteria, and epithelial cells lining the human mouth, gives greater contrast, more detail, and easier viewing. Lugol's iodine is useful in staining thin slices of onion to visualize the nucleus. Methylene blue (as a vital stain) is often used to stain both the cytoplasm and nucleus of epithelial cells from inside the mouth (see Table 1).

Table 1: Common Stains Used in Microscopy

I. Methylene Blue: may be used as a vital or bacterial stain.



II. Lugol's Iodine

10g potassium iodide dissolved in 100 mL of water
5g iodine added slowly while mixing
Filter and store in a brown bottle.

III. Gram Stain Reagents

1. Crystal Violet
10g crystal violet
500 mL absolute methanol

2. Gram's Iodine
6g iodine
12g potassium
1800 mL distilled water
Store Gram's iodine in dark bottles.

3. Acetone-Alcohol or Alcohol Decolorizer
A mixture of equal volumes of acetone and 95% ethanol is the preferred decolorizer. Ninety-five percent ethanol may be used instead.

4. Safranin
10g safranin
1000 mL distilled water

Most stains are effective only after the cells are dead and dehydrated. One drop containing the living specimen suspended in a liquid is added to a slide and allowed to completely air dry (heating at this point tends to shrink and distort the appearance of the specimen). The slide is then heat fixed by passing the slide rapidly through an open flame several times (see Table 2). Slides can be concomitantly air dried and heat fixed by using a warming tray. Heat fixing kills the specimen by denaturing its protein and attaches the coagulated protein of the specimen to the slide (some very hardy pathogenic bacteria, such as the mycobacteria that cause tuberculosis, are not killed by heat fixing). Instead of heat fixation, chemical fixation, in which a drop of methanol, osmic acid, glutaraldehyde, or formaldehyde is added to a drop of the specimen, may be used to preserve delicate specimens. Once the slides are fixed, they are ready to be stained.

Specimens can be preserved by either chemical or heat fixation.

Dyes are classified as either acidic or basic. Acidic dyes (such as eosin, acid fuchsin, and congo red) react with the cell wall, proteins, and cytoplasm. Because bacterial surfaces are negatively charged, they are more easily stained with basic dyes. Methylene blue, crystal violet, safranin, and basic fuchsin are basic dyes commonly used in biological science laboratories. These dyes stain nucleic acids and some polysaccharides. Dyes may be used in a simple staining procedure, in which only one stain is applied to the specimen. With simple stains, all cells appear the same color, but the shape, size, arrangement, and nucleus of eukaryotic cells are readily apparent.

Acidic dyes react with the cell wall, proteins, and cytoplasm, while basic dyes react with bacterial surfaces.

Dyes may be used in differential or special stains, in which two dyes (primary stain and counterstain), sometimes in conjunction with a mordant or decolorizer, are applied to give a particular reaction. The most important differential stain for bacteria is the gram stain (see Tables 1 and 2), in which gram-positive bacteria stain purple and gram-negative bacteria stain red due to a fundamental difference in cell wall structure. Another differential stain is the acid-fast stain. (Acid-fast organisms are red and non-acid-fast organisms are blue-green.) Clinically, it is used to detect the acid-fast bacterium, *Mycobacterium tuberculosis*, from the sputum of patients with tuberculosis. In the differential capsule stain, flagella stain, and endospore stain, presence or absence of these bacterial cell features can be noted. Wright's stain is a differential stain for distinguishing the types of blood cells, while hematoxylin-eosin stain is useful for tissue slides.

The gram stain divides bacteria into two groups:

- 1) *gram-positive*
- 2) *gram-negative.*

Wright's stain is used to differentiate types of blood cells.

See NABT's Policy Statement on the use of body fluids.

Table 2: Heat Fixing and Gram Staining

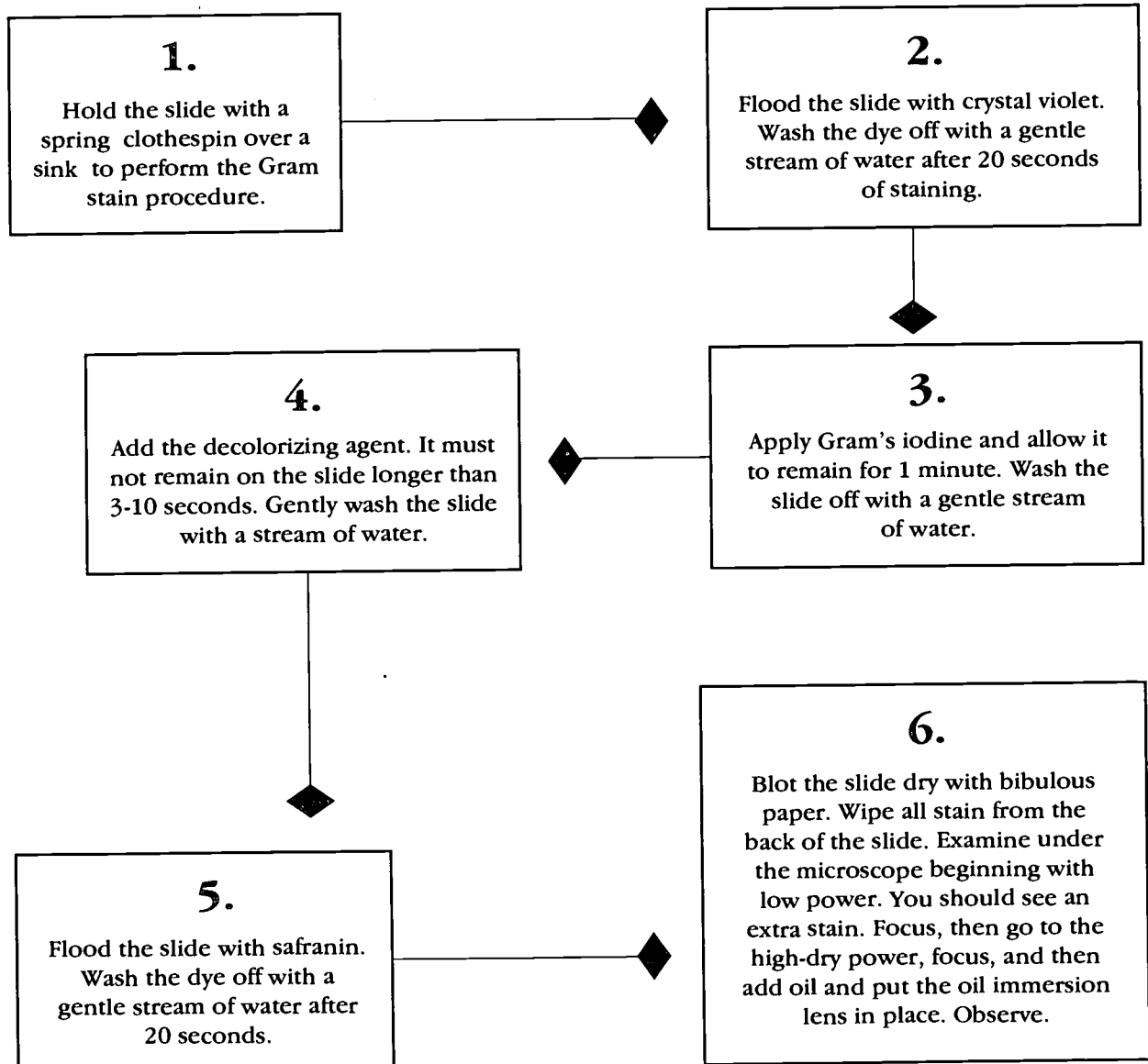
Heat Fixing

Hold the end of the slide with a spring clothespin. Pass the slide quickly (1-2 seconds) through the hottest part of the flame of a Bunsen burner or alcohol burner. Repeat this three times.

CAUTION — *Do not hold the slide in the flame because the heat will break the slide!*

The slide is now ready for gram staining.

Gram Staining



Examples of specimens useful for introductory classroom microscopy include:

- 1) newspaper print
- 2) colored thread
- 3) molds
- 4) pond water
- 5) hay infusion
- 6) raw onion
- 7) cheek cell scrapings

Specimens for microscopy can be easily obtained. Commercial biological companies sell small quantities of many protozoans, algae, bacteria, and fungi (as well as microscopes and stains), but everyday materials can make wonderful specimens. Small pieces of newspaper print, colored cotton or wool threads, and leaflets of the aquatic plant *Elodea anacharis* make good specimens (*Elodea* leaflets are two to three cells in thickness). A moldy piece of bread or orange can supply an entire class with observation material. Pond water that includes a few submerged leaves and small floating plants, if allowed to sit for several days, will probably contain protozoans and invertebrates for study. Easily prepared hay infusions (two quarts of pond water and a handful of dry grass or hay, allowed to sit for two weeks) usually yield *Paramecium*, nematodes, fungi, and bacteria.

A raw onion can be used to make good preparations of plant cells that can be viewed without stain, or with methylene blue or Lugol's iodine stain (see Table 1). To prepare the wet mount, slice the onion thinly and cut a single ring into one-quarter inch sections. With forceps, remove the inner transparent membrane and put the membrane into a small drop of water on a slide. Add a coverslip and observe. Then stain and observe.

Thin smears of non-pathogenic bacteria are easy to make and stain. Gently scrape the lining of the mouth inside the cheek surface and the surface of a tooth using the flat end of a toothpick. Mix the material from the toothpick in a drop of water on a microscope slide. You can observe the specimen as a wet mount, or you can allow the slide to completely air dry, then heat fix, and gram stain it (see Table 2). Gram-stained epithelial cells are pink with a darker pink nucleus. Using the oil immersion objective, some small, round, purple-stained bacteria (some in long chains) are commonly seen. Red, round bacteria and a few red rod-shaped bacteria may also be seen. These smears will allow students to make size comparisons between body cells and bacteria.

Bacteria are easily grown using dried beans. Place about one-fourth cup dried beans in a leak-proof cup containing one cup of water. Allow the beans to soak for two days. Remove a drop of the water surrounding the beans and place it on a microscope slide. Bacteria observed come from the endospores that survive on the surface of dried plant materials. These bacteria are motile and are large enough to see under high-dry po-

wer. They make a good specimen for hanging-drop wet mounts as well as stained slides. If a Gram stain is desired, allow the drop to completely air dry. Then heat fix and Gram stain it (see Table 2). Most bacteria observed in the Gram-stained preparation are purple-blue rods, many in serpentine chains, while some are red rods.

Guidelines for Viewing a Specimen with a Compound Light Microscope

1. Clean oculars and objectives using lens paper. For microscopes with a built-in variable illuminator, plug in the microscope and turn the voltage to half of its maximum. For microscopes with a mirror, adjust the mirror to receive the maximum light from room light or a free standing lamp. Open the iris diaphragm and rotate the lowest power objective into place.
2. Center the slide over the hole in the stage and secure it, using stage clips to hold the slide to the stage.
3. Use the coarse focus knob to bring the stage and objective as close as possible to each other without actually touching. While looking through the ocular(s), use the coarse focus knob to *increase* the distance between the objective and stage until the specimen comes into focus. Center the specimen in the field of view.
4. Use the fine focus knob to sharpen the focus the specimen.
5. Adjust the light intensity until it is optimal for viewing at the magnification being used. (Keep the iris diaphragm open for stained specimens.)
6. Record your observations and make pencil drawings, as necessary. When drawing from microscope observation, visualize the field of view as being a circle 1.5-2.0 inches in diameter. Draw the specimen's size in proportion to the size of the field of view. Draw what is observed and include details, not just the outlines of the object. If the specimen has color, add color with colored pencils.
7. To change to a higher power objective, rotate the nosepiece and click the next objective into place. Repeat steps 4 through 6, if your microscope is parfocal. Repeat steps 3 through 6, if your microscope is not parfocal.

8. To use the oil immersion objective, rotate the nosepiece to swing the high-dry objective out of place. Before rotating the oil immersion objective in place, add a drop of immersion oil to the area of the slide to be viewed and click the objective in place. Repeat steps 3 through 6 for a non-parfocal microscope, 4 through 6 for a parfocal microscope.

CAUTION: ALWAYS "COARSE FOCUS" ON THE LOWEST POWER OBJECTIVE. YOU SHOULD NOT COARSE FOCUS AGAIN (EVEN WHEN CHANGING OBJECTIVES) WHEN USING A PARFOCAL MICROSCOPE BECAUSE OF THE DANGER OF DAMAGING OBJECTIVE OR SLIDE.

Variations of Bright-Field Microscopy

Stereoscopic or Dissecting Microscopy

Stereomicroscopes are used to view opaque specimens at low magnifications (10-40X).

The stereomicroscope (stereoscope) is easy to use and maintain. It is best for viewing opaque specimens using low magnifications (10-40X). Models have either an opaque or transparent stage plate, two oculars, and a focus knob. The most useful stereomicroscopes have built-in illuminators, one producing reflected light and one producing transmitted light. Because it produces a three-dimensional real image, it is the best type of microscope for viewing small plants and animals in the living state, such as *Daphnia*, *Hydra*, *Planaria*, seeds, ferns, mosses, and flower parts. The long working distance between stage and objective allows for viewing of specimens in petri and culture dishes, and for viewing surface features of both plants and animals during dissections.

Dark-Field Microscopy

Dark-field microscopy uses a special substage condenser that causes light waves to be diffracted around the specimen rather than through the specimen.

Dark-field microscopes were designed to increase contrast and resolution of the light microscope. A special dark-field condenser (or the insertion of a dark-field stop into a condenser) is used to block all light from the center of the condenser. The field of view appears black, and the specimen appears to glow because the condenser stops all light from entering the objective except the light reflecting or scattering off the specimen (see Figure 6-2). Dark-field technology has been successful in viewing microorganisms with diameters of 0.1-0.2 micrometers (below or at the limit of light microscopy resolution) because the organisms appear to emit light and, thus, are visible. This type of microscope provides contrast in living, unstained cells and organisms though it shows less internal detail than phase-con-

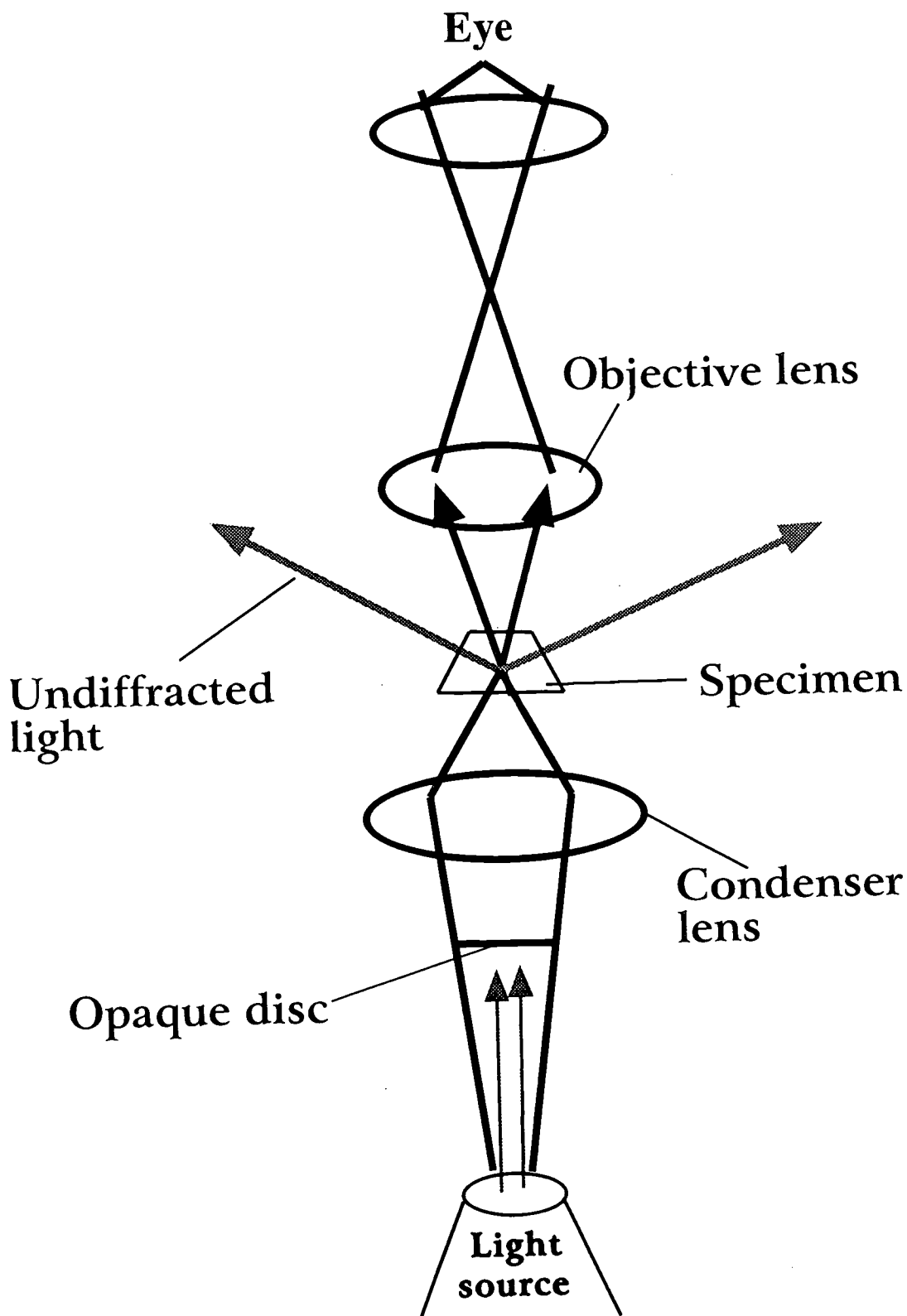


Figure 6-2

Basic schematic outline of the dark-field microscope.

trast microscopy does. In the medical laboratory, dark-field microscopy is important in the identification of *Treponema pallidum*, the bacterium causing syphilis; *Borrelia burgdorferi*, the agent of Lyme's disease; and *Leptospira*, the cause of leptospirosis.

Inverted Microscopy

Inverted microscopy allows for the viewing of specimens at the bottom of a flask or container.

The inverted microscope has its parts positioned in inverted order from the bright-field microscope. The source of transmitted light is located at the top, while the objectives rotate around a nosepiece situated underneath the stage. This arrangement gives a large distance for specimen placement. The inverted microscope allows one to examine a specimen on the bottom of a container at high magnification. Uses of the inverted microscope include observations of tissue culture cells in growth flasks, roller bottles, or dishes, and examinations of sediments, precipitates, and reactions seen in microtiter plates.

Phase-Contrast Microscopy

Phase-contrast microscopy facilitates the study of unstained live specimens.

It is difficult to study cellular organelles in the living state with bright-field microscopy because most cytoplasmic structures are completely transparent. Stained slides of dead cells permit the study of artifacts of the organelles, not the actual *in vivo* structures. The phase contrast microscope is able to differentiate some cellular organelles without stains and in the living state. Phase-contrast microscopy translates the varied densities in cell organelles and their accompanying refractive indexes into differences in light intensity. Light traveling through a dense material moves more slowly than light traveling through a less dense material. The slower, diffracted rays are one-fourth of a wavelength slower than the direct rays. Light rays from the specimen are out-of-phase with each other. If the direct rays are slowed down one-fourth wavelength, direct and diffracted rays will give an additive effect that is proportional to the square of the amplitude. Thus, the brightness or contrast is increased four times. The major structural difference between bright-field and phase contrast microscopes is the placement of special opaque rings in the objective lens and condenser of the phase-contrast instrument.

Nomarski or Differential Interference Contrast Microscopy

George S. Nomarski, in the late 1950s, expounded the

principles that laid the foundation for development of the Nomarski or differential interference contrast (DIC) microscope, which has come into use during the recent past. It is a type of phase-contrast microscope that places prisms rather than opaque rings in the objective lens and condenser. Prisms split each light beam, giving contrasting colors and an almost three-dimensional image to the specimen. Greater internal detail and more vivid images of unstained, live specimens are produced by the DIC than other phase-contrast microscopes. Topographic features of specimens viewed by DIC are similar to the quality of specimens viewed by scanning electron microscopy (SEM) at lower magnifications.

Other Types of Compound Light Microscopes

Fluorescent Microscopy

The fluorescent microscope uses ultraviolet light to view specimens that fluoresce.

The fluorescent microscope uses light of very high energy. Fluorescent microscopy is dependent upon a specimen being illuminated with one wavelength of light and emitting light of another wavelengths. Short, invisible, high-energy wavelengths, usually in the ultraviolet range, are the illuminating or excitation wavelength, while visible wavelengths are the emission wavelengths. Most organisms and cells do not fluoresce on their own, but can be coated with a fluorescent dye or fluorochrome. The fluorescent cell glows an intense red, orange, or yellow against a black background. The fluorochrome acridine orange becomes incorporated into nucleic acids where it fluoresces green in mammalian cells and orange in bacterial and yeast cells. The combination of auramine and rhodamine dyes is used to visualize mycobacteria, which fluoresce yellow.

Fluorescent microscopy utilizes three filters not needed for bright-field microscopy (see Figure 6-3). Between the ocular and objective, an absorbency filter or barrier is placed to block harmful levels of ultraviolet rays from entering the eye. Blindness could result from the absence of this filter. Two filters must be used between the specimen and the lamp. A narrow bandpass exciter filter, appropriate to the fluorochrome used, bombards the specimen with short wavelengths near the ultraviolet range. The second filter, a stop filter, transmits visible light to the objective, while blocking ultraviolet light. Some fluorescent microscopes use a special dark-field condenser to produce a dark background and to help achieve maximum resolution.

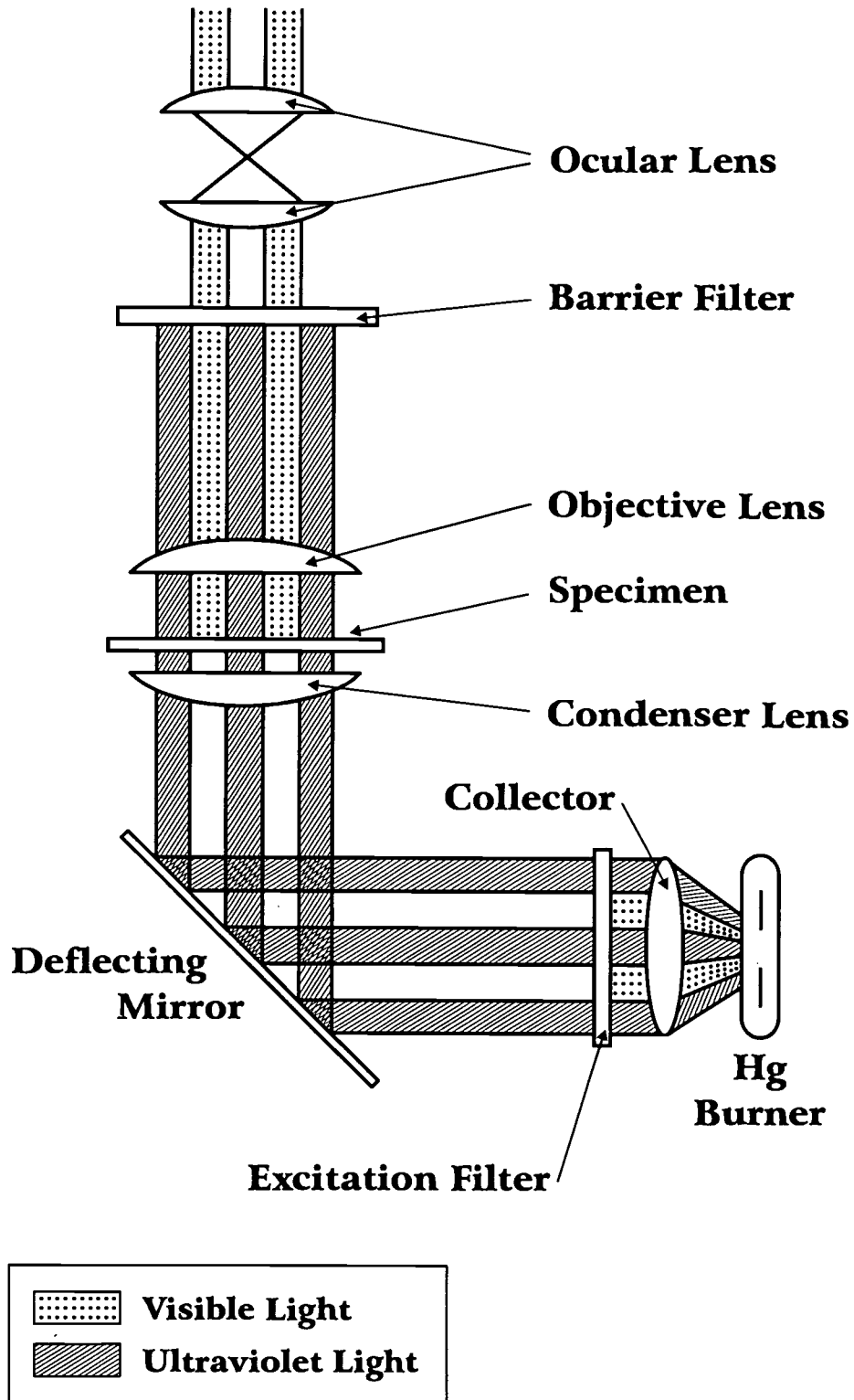


Figure 6-3
Principle of fluorescent microscopy.

Transmitted fluorescent microscopy employs ultraviolet light from below the specimen to improve the intensity of the fluorescence.

If the ultraviolet light comes from below the specimen, it is termed transmitted fluorescence. When the ultraviolet light enters through the objective lens, the technique is termed *epifluorescence*. With transmitted fluorescence microscopy, the intensity of fluorescence decreases exponentially with the total magnification. One advantage of epifluorescence is that, as magnification is increased, the intensity of fluorescence increases.

Two types of lamps may be used in the excitation lamp — mercury or tungsten-halogen. The mercury vapor lamp generates high-intensity ultraviolet, violet, and blue light. The lamp is housed in a special compartment to guard against damage and help in heat dissipation. Mercury lamps must be maintained in strict accordance with the manufacturer's regulations and not exceed rated hours of usage or the bulb may explode. Tungsten-halogen bulbs give many hours of viewing, and are not explosive, but do not offer quite the intensity of a mercury bulb.

Fluorescent microscopy can be used to diagnose infections resulting from certain bacteria, protozoans, and viruses by using different fluorescent antibody techniques.

Fluorescent microscopy is invaluable in diagnosing infections caused by certain bacteria, protozoans, and viruses by using antibodies tagged with a fluorochrome. These tagged antibodies react specifically against microbes present in clinical samples. One use of fluorescent antibody technology is the detection of the autoimmune disease, *systemic lupus erythematosus*. People with this disease produce antibodies against the nuclei of their own cells.

These antibodies are detectable by the anti-nuclear antibody test, using fluorescent microscopy. A sample of the patient's serum is added to nucleated cells on a microscope slide, incubated, washed, then flooded with a specially-prepared antibody (anti-human globulin) tagged with a fluorescent dye. After incubation (in which complementary antibodies bind) and a thorough washing of unattached antibody, the slide is viewed by fluorescent microscopy. Intense fluorescent coloration of the cells' nuclei indicates a positive test for anti-nuclear antibody.

Confocal Scanning Microscopy

Confocal scanning microscopy requires a laser to generate a single beam of intense light, lenses of excellent numerical aperture, and a computer to create images from the detected light. Commonly used lasers are argon-ion, argon-krypton, helium-neon, and helium-cadmium. Which laser to use depends upon the application of the microscope. Laser light images generated

with this microscope are viewed on a monitor, rather than directly through oculars. Direct viewing would damage the eyes.

Confocal imaging permits the observer to visualize a single plane of focus or combine serial sections into a 3-D view.

Confocal microscopy produces greater resolution than traditional light microscopy and displays the image on a monitor screen.

Confocal microscopes are used to view:

- 1) *certain ions in cells such as calcium ions in cardiac tissue;*
- 2) *antibodies, receptor proteins, and neuron activity;*
- 3) *toxins or drugs in living tissue; and*
- 4) *microcirculations.*

Confocal microscopy can be used to view thick specimens — allowing for examination of whole organisms.

A three-dimensional image that is completely in focus is produced. The laser is focused to a point by an objective lens. Two mirrors distribute this light beam across the specimen. The second objective lens magnifies the image, while light from only a specific focus point is detected, eliminating all diffracted light. In effect, this microscope creates an image without distortion. The lack of distortion enables this microscope to achieve greater resolution than is possible with other types of light microscopes.

Confocal microscopy can be used to view certain ions within cells. Calcium ions can be seen in channels of heart muscle cells when an argon ion laser and fluorescent dyes are used. Concentrations of hydrogen ions within cells can be measured using fluorescent dyes and confocal imaging. Antibodies, receptor proteins, and neuron activity can be localized by the use of special lasers, fluorescent dyes, and probes. Living animals, including humans, can be examined by confocal scanning microscopy. The effects of toxins or drugs in tissues up to 300 μm below the surface can be investigated. The microcirculation on rat-brain and blood vessels have been studied by confocal microscopy. In living animals, prolonged exposure to the high-intensity laser light must be avoided.

Conventional confocal microscopy generates images of good resolution with good sensitivity. Fluorescent dyes used with an appropriate laser allow one to view some metabolic processes of a cell or organism. Newly-designed fiberoptics make the microscope miniaturized and portable. A modification of confocal scanning microscopy uses a conventional microscope equipped with a xenon or mercury light source rather than laser illumination. Use of these alternate energy sources eliminates cellular destruction caused by specific lasers.

Confocal scanning microscopy has proven itself to be an indispensable research tool. With it, one can view the living cell and see dynamic metabolic processing and the functioning of body tissues. This system can view relatively thick specimens (up to 40 μm), which allows one to examine whole organisms and embryos of many species. Epifluorescence coupled with confocal scanning microscopy has enabled researchers to pinpoint actual locations of metabolic functions within cells and tissues.

IC Inspection/Metallurgical Compound Microscopy

The IC inspection/metallurgical compound microscope is highly specialized for industrial applications of inspecting integrated circuits, wafers, electronic components, and metals. Illumination can be provided by tungsten halogen, higher wattage halogen, mercury, or xenon bulbs. The microscope may be modified by phase optics or internal differential interference contrast prisms. Biological applications are possible.

Combination Microscopy

Today, many research and clinical laboratory microscopes serve more than one function. Many compound light microscopes have phase optics, Nomarski differential phase-contrast optics, as well as fluorescent and dark-field capabilities. Inverted light microscopes are often fluorescent microscopes, too. These combination microscopes save time, money, and lab bench space.

Projected and Video Microscopy

A new microscopic technique is video-enhanced microscopy. This technique uses objectives and condensers with the highest possible numerical apertures. A metal halide illuminator creates a completely even light field which is transmitted over fiber optic bundles. Every aperture is fully illuminated, giving superior resolution, but no contrast. The naked eyes would be able to see nothing. Artificial contrast is provided by way of video enhancement circuitry. Even live specimens can be examined using Nomarski optics. With this technique, the limit of resolution approaches the theoretical limit of 200 nm.

A *microprojector* may prove helpful for teaching students in a setting in which individual microscopes are not available. Images produced may be projected onto a sheet of paper below the projector or onto a screen for the entire class to observe. Low power observations can be made using wet mounts of live, actively moving protozoans and colorful algae. Microprojection requires a darkened room, the darker the better. Lack of both contrast and resolution limit the usefulness of this tool.

A video microscope is a more sophisticated tool than a microprojector. Typically, a video camera is attached to a trinocular microscope. A video camera is attached to the microscope and the microscope image is displayed on a TV screen. Resolution is best when the specimen is viewed using low and high-dry magnifications. Video microscopy is a useful teaching

tool because the students are all looking at the same specimen together. It can be used as a preview before students actually perform a lab exercise, as a review of lab exercises, or to give practical exams. Video microscopy is also helpful when only a limited sample is available for class examination. Physiological activities in progress can be visualized by the class as a whole as the teacher points out plasmolysis, cytoplasmic streaming, or the functioning of contractile vacuoles. Video microscopy can be used to produce a videotape of the specimen(s) seen through the microscope. The tape can then be used in place of, in conjunction with, in review of, or in evaluation of a wet lab.

Photomicroscopy

Superior optics produce superior photographs. *Aplanatic-achromatic* or *apochromatic objectives* give consistently good results. Alignment of lenses, including the condenser, and appropriate illumination become critical details for micro-photography. Optimum output from a highly resolving objective depends upon optimum illumination. Koehler illumination provides the best light for photography. Koehler illumination is defined as "an aligned light source, focused oculars, and stopped down field and iris diaphragms." The Abbe condenser is then centered and focused, and diaphragms opened. In Koehler illumination, the light is focused on the condenser rather than on the specimen.

Neutral density filters may be used to help control the brightness of the lamp, and color filters can restrict the wavelength of light used. Red or orange Wratten filters enhance structures stained with blue dyes, while green filters improve the contrast of red-stained structures. Interference filters of a narrow band-pass are also available, but more expensive than Wratten filters. The filters used with black-and-white film depend upon the color of stain in the specimen, with the aim of producing good contrast in the photograph. Cameras used for photomicroscopy can be either the 35 mm or 4 x 5 large format type. Thirty-five millimeter cameras are the most commonly used type for color photographs, while the 4 x 5 format Polaroid camera is most commonly used for black-and-white photography.

Guidelines for Better Microscopy

1. Purchase the highest quality microscope you can afford for the job at hand. Parfocal, achromatic lenses are always good

purchases. A spring-loaded nosepiece that retracts to protect the objectives lessens the chances of accidental breakage of the objective during use. The use of a binocular microscope is less tiring than the use of a monocular microscope.

2. Built-in light sources provide good, consistent illumination. If your microscope has a mirror instead of a lamp, do not position the mirror to receive direct sunlight. Direct sun rays hitting your eye can cause burning of the retina.
3. Purchase good quality slides and cover slips. Pre-cleaned slides of 1.24 mm thickness are best for detailed microscopy, and frosted-end slides are good for labeling slides kept for future reference. Coverslips for low power magnifications may be either plastic or glass, but glass is preferable for high-dry work. (Plastic coverslips are easily scratched and should not be reused.) A good quality glass coverslip, No. 1°, 0.17 - 0.19 mm thickness, is needed for best resolution in oil immersion work.
4. When using a monocular microscope, view with both eyes open even though you are using only one eye. Do not squint or close the unused eye, Until that eye begins to ignore what it sees, you may place a hand over it like a patch.
5. Always carry a microscope with both hands. One hand should be placed under its base, the other hand around its arm (see Figure 6-1.)
6. Take good care of your microscope. Always place a dust cover over it when not in use. Wrap the cord loosely around the base. Clean oculars and objective lenses before and after use (at least daily) with a good quality lens paper. A commercial lens cleaning fluid applied to lens paper should be used as needed. Do not soak objectives in these cleaning fluids, as they can loosen the seal of the objective and seep under it. You can tell if the oculars are dirty by rotating the ocular while looking through it. If the speck of dirt rotates, the ocular needs cleaning (mascara, eyelashes, and oil from fingers are often responsible for dirtying oculars).
7. When using the oil immersion objective, use an immersion oil of a medium high viscosity (type B is recommended for routine use). One drop of oil is enough; oil left on the microscope attracts dust and should be removed after use.
8. Do not use the condenser to adjust light intensity. The best

position for the condenser is flush with the stage. This upper-most position gives the best resolution when using the oil immersion objective.

9. Objective lenses and the focus knobs will correct for near- and farsightedness, so glasses prescribed for these conditions can be removed when doing microscopy. Microscope lenses do not correct for astigmatism, so glasses must be worn if prescribed for this condition.
10. As magnification is increased, the need for light increases. Open the iris diaphragm to increase the light. When the iris diaphragm is open and more light is needed, turn up the lamp voltage if this is possible on your microscope.
11. Have your microscope professionally cleaned and serviced as need dictates (once a year if the microscope is used regularly). Replacement of lamp bulbs can be done in-house, using the instructions provided with the instrument.
12. Bulb life on microscopes with variable voltage can be increased by not turning the voltage to its maximum unless absolutely necessary. Make sure the iris diaphragm is completely open before increasing voltage.
13. If you lose the specimen in going from low power to high-dry power, go back to the low power objective, center the specimen in the field of view, fine focus, then switch to the high-dry power. If you lose the specimen going from high-dry to the oil immersion power, wipe all oil from the slide and begin again with low power. Be sure the specimen is in focus at each magnification before advancing to a higher power.
14. The use of coverslips helps increase resolution. Coverslips must be used to cover wet mount preparations but are not necessary to cover dry and stained, non-permanent slides to be observed with the oil immersion lens.

Questions Commonly Asked by Students

Question 1: Why is the magnification of a light microscope limited to 1500X?

Most light microscopes can effectively magnify only one thousand times. Only microscopes with the best available optics are able to provide more detail when the magnification is increased to 1500X.

The wavelength of our energy source, light, and the construction of the optics (oculars, objectives, and condenser) define and limit the resolution of the microscope. When we magnify beyond this limit of resolution, the image becomes blurry rather than clearer. We call this type of magnification "empty magnification" since it is useless.

Question 2: When, after high school, will I ever need to know about the microscope?

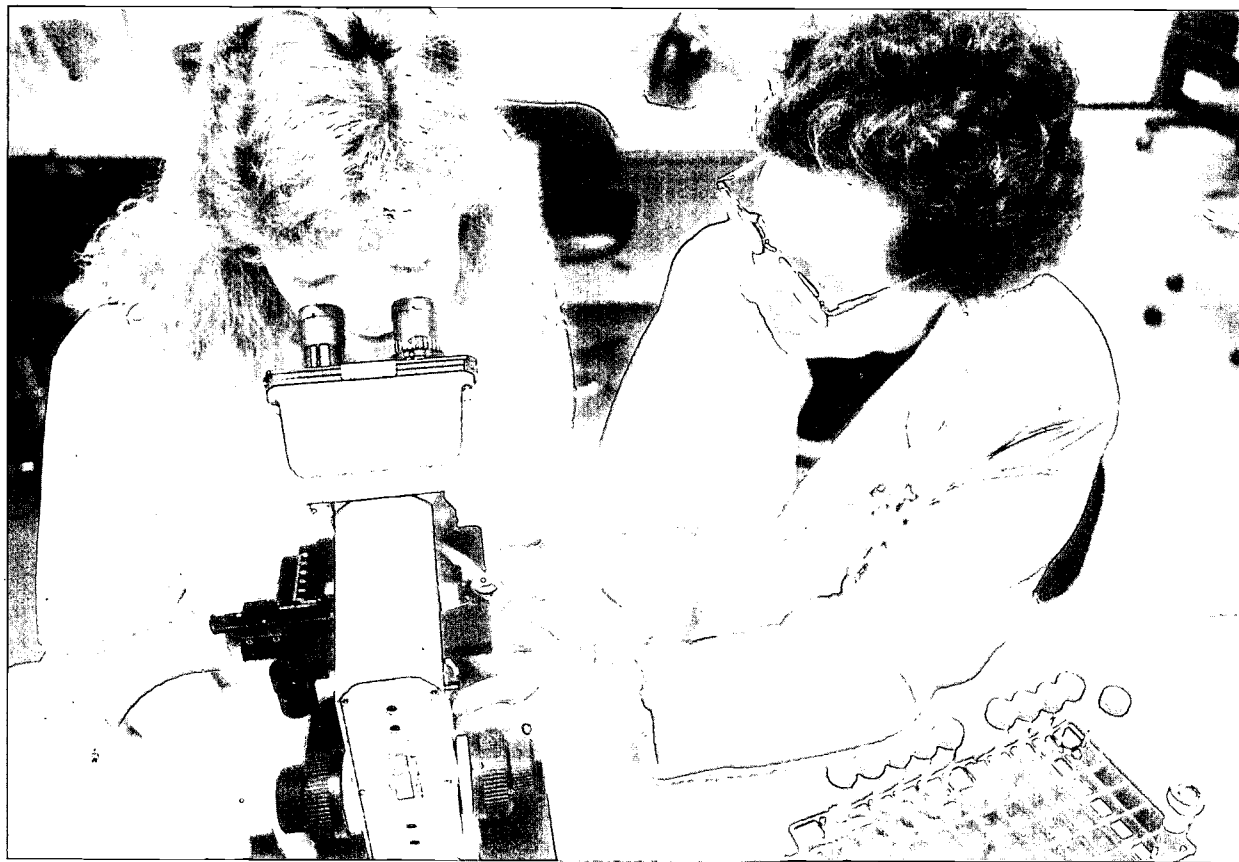
For students attending college, knowledge of the microscope is required. Most life science courses, such as biology, anatomy, histology, genetics, microbiology, zoology, and physiology have a laboratory portion that will necessitate use of a microscope.

Teachers, medical assistants, laboratory technologists, food scientists, marine biologists, electrical engineers, physicists, doctors, nurses, veterinarians, dentists, and biologists must be knowledgeable of the microscope and its uses.

Question 3: What are the advantages and disadvantages of the dissecting microscope and the compound light microscope?

The dissecting or stereoscopic microscope produces a three-dimensional image and is ideal for viewing small, live specimens. It has a large working distance and can accommodate dissecting pans, petri dishes, and culture dishes. Its main disadvantage is the low magnification it can produce (40X). The compound light microscope images in two-dimensions and the im-

age it produces is upside down and backwards. It can only be used to view very thin, transparent specimens, while it can image some living cells and organisms. Most specimens are dead and have been sliced before they can be viewed. Most compound light microscopes can only magnify to 1000X. Both the dissecting and compound microscopes are relatively affordable and portable.



Dr. Cox instructs Rhonda Blankenship, a junior chemistry major at Louisiana State University at Shreveport, in the proper use of the light microscope.

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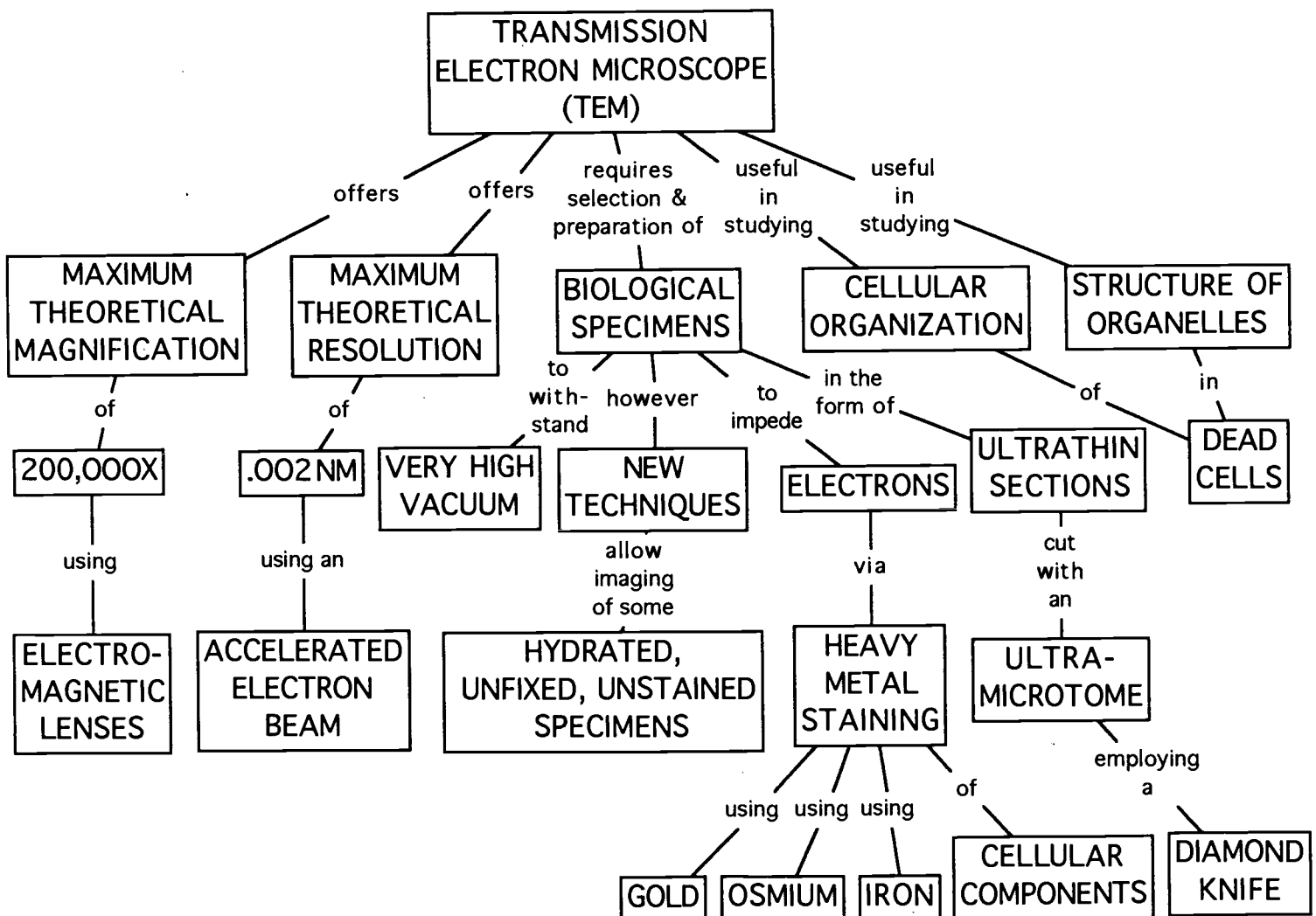
TRANSMISSION ELECTRON MICROSCOPY

7

Jerry A. White and Merton F. Brown

Overview

1. Comparison of light and transmission electron microscopy (TEM)
2. Concepts of wavelength, magnification, and resolution
3. Basic operation of the TEM
4. Preparation of biological specimens for the TEM
5. Advanced techniques for the TEM



Transmission Electron Microscopy

There are two basic types of electron microscopy: transmission and scanning.

Until the 1950s, imaging of ultracellular structure was confined to light microscopy. This form of imaging was limited and remains limited in terms of magnification and resolution. Improvement in light microscopy has been aimed at improving the contrast of the viewed specimen. During the early 1950s, the introduction of electron microscopy provided phenomenal advances in the ability to see and understand cell ultrastructure. Although most biology textbooks are abundantly illustrated with electron micrographs, the majority of biology teachers have not had an opportunity to gain firsthand experience with the electron microscope. While a written description is no substitute for hands-on experience, we hope that the following discussion will provide teachers with a better understanding of electron microscopy. This chapter discusses transmission electron microscopy (TEM). Scanning electron microscopy (SEM) will be described in the following chapter.

Resolving Power and Magnification

Resolution refers to the ability to distinguish between two adjacent image points.

While most people think of the electron microscope as an instrument capable of high magnification, its real strength lies in high *resolving* power. Resolving power or resolution is defined as the ability of an optical system to distinguish two closely-spaced points as separate objects. If the two points are closer together than the resolving power of the instrument, they will appear as one point. Resolving power is determined by measuring (usually on a micrograph taken at high magnification) the space between these two close, but distinctly separate, image points.

A nanometer is one billionth of a meter

In 1932, the German electrical engineer Ernst Ruska invented the electron microscope.

At the beginning of this century, physicists declared that the light microscope had reached the limit of magnification and resolution capability. They determined that the resolving power of any microscope was limited by the effects of diffraction to about one-half the wavelength of the illumination (light source) used. Therefore, the best resolution that could be achieved with the light microscope was approximately 0.2 μm (200 nm). Clearly, the only solution to this problem was to utilize shorter wavelength illumination. During the mid-1920s, physicists discovered that a beam of electrons possessed an extremely short wavelength and that such a beam could be focused by passing it through a magnetic field. Although these discoveries laid the foundation for electron optics, the first useful electron microscope was not developed until the early 1950s.

TEM limitations:

- 1) *diffraction*
- 2) *spherical aberration*
- 3) *chromatic aberration*
- 4) *astigmatism.*

The modern electron microscope uses an electron beam having a wavelength of about 0.004 nm. This yields a theoretical maximum resolving power of 0.002 nm. Unfortunately, a number of inherent physical limitations, such as diffraction, spherical aberration, chromatic aberration, and astigmatism serve to degrade instrument performance. In actual practice, modern, state-of-the-art TEMs are capable of resolving 0.2-0.3 nm using ideal test specimens. However, with thin-sectioned biological specimens, a more realistic figure is 3-4 nm.

Useful light microscope magnification is limited to 1000-1500 times. TEM magnification is about 200,000 times.

With any microscope, the human eye is the ultimate image recording device. Because of the structure of the retina, the unaided eye has a practical limit of resolution of about 0.2 mm. The only way for the eye to appreciate the resolving power of a microscope is to have the object magnified so that the image seen by the retina is larger. The magnification needed in order to fully utilize the resolving power of the light microscope is about 1000 times. Obviously, if the light microscope is capable of 0.0002 mm resolution, then in order to reach the 0.2 mm resolving power of the eye, this level of magnification is necessary. In the case of the TEM, for the eye to resolve 1.0 nm detail in the microscopic image, a magnification of 200,000 times is required. However, since the image is recorded on film and later printed, the photographic enlargement factor (usually 3-4 times) means that the microscope magnification does not need to be pushed to the theoretically required level.

The relationship between wavelength, resolution, and magnification governs the practical effectiveness of microscopy.

With any type of microscope, however, it is important to recognize where useful magnification ends and "empty" magnification begins. There is no technical difficulty in building a microscope that will magnify in excess of the resolution provided by that particular type of instrument. In the case of the light microscope, magnification in excess of 1000 times results in no additional detail and hence is *empty* magnification. No combination of lenses can overcome the physical limitations imposed by the wavelength of visible light. It is this relationship between wavelength, resolving power, and maximum useful magnification that is the basis for the electron microscope's unique capabilities.

The Basic Electron Microscope

The transmission electron microscope is a complex arrangement of electronic and mechanical components. However, for the sake of simplicity, the TEM can be described as being

composed of five basic systems: (1) illumination system, (2) image forming system, (3) specimen positioning system, (4) image recording system, and (5) the vacuum system (see Figure 7-1 and Appendix A).

A large voltage differential propels electrons down the TEM column at a high velocity.

An illumination system consists of a cathode-anode electron gun and associated electronics. The gun is located at the top of the microscope column where it produces a beam of electrons. The beam is produced by heating a V-shaped tungsten wire filament to a temperature sufficient to drive off electrons from the filament tip. This cloud of electrons is drawn toward an aperture in the anode plate by a high voltage differential that exists between the cathode (the filament) and the anode. The anode is held at ground potential while the cathode shield is held at a high negative potential (-50 to -100 kv). This large voltage differential propels the electrons down the column at high velocity.

The basic TEM employs three lenses to produce an image:

- 1) *electromagnetic lens*
- 2) *condenser lens*
- 3) *objective lens.*

The image forming system consists of electromagnetic lenses that serve the same function as glass lenses in the light microscope. Each lens is capable of producing a controlled magnetic field within a small-diameter central bore. The electron beam passes down through this bore, where it is acted upon by a magnetic field. Varying the lens current allows the operator to vary the magnetic field and thus control electron beam deflection. The result is an electron beam that can be shaped and focused on a given plane. In a basic TEM, three lenses produce the image. A condenser lens shapes the beam and focuses it on the specimen, an objective lens produces the first magnified image of the specimen, and a projector lens produces the final high magnification image. In actual practice, the modern microscope typically has two condenser lenses, one objective lens, and two or three projector lenses. The specimen, usually an ultra-thin section of tissue, is placed in the path of the beam at the level of the objective lens (see Appendix A). The final magnified image is projected onto a viewing screen at the bottom of the column. This screen is coated with a substance (heavy atoms of a metal) that fluoresces when struck by electrons. The image then is viewed with a 10-power binocular optical microscope or recorded on photographic film.

The specimen positioning system consists of the specimen stage and its mechanically linked controls which allow the operator to move the specimen in the X and Y directions. New, state-of-the-art microscopes that are computer-controlled use motor-driven, multi-axis stages.

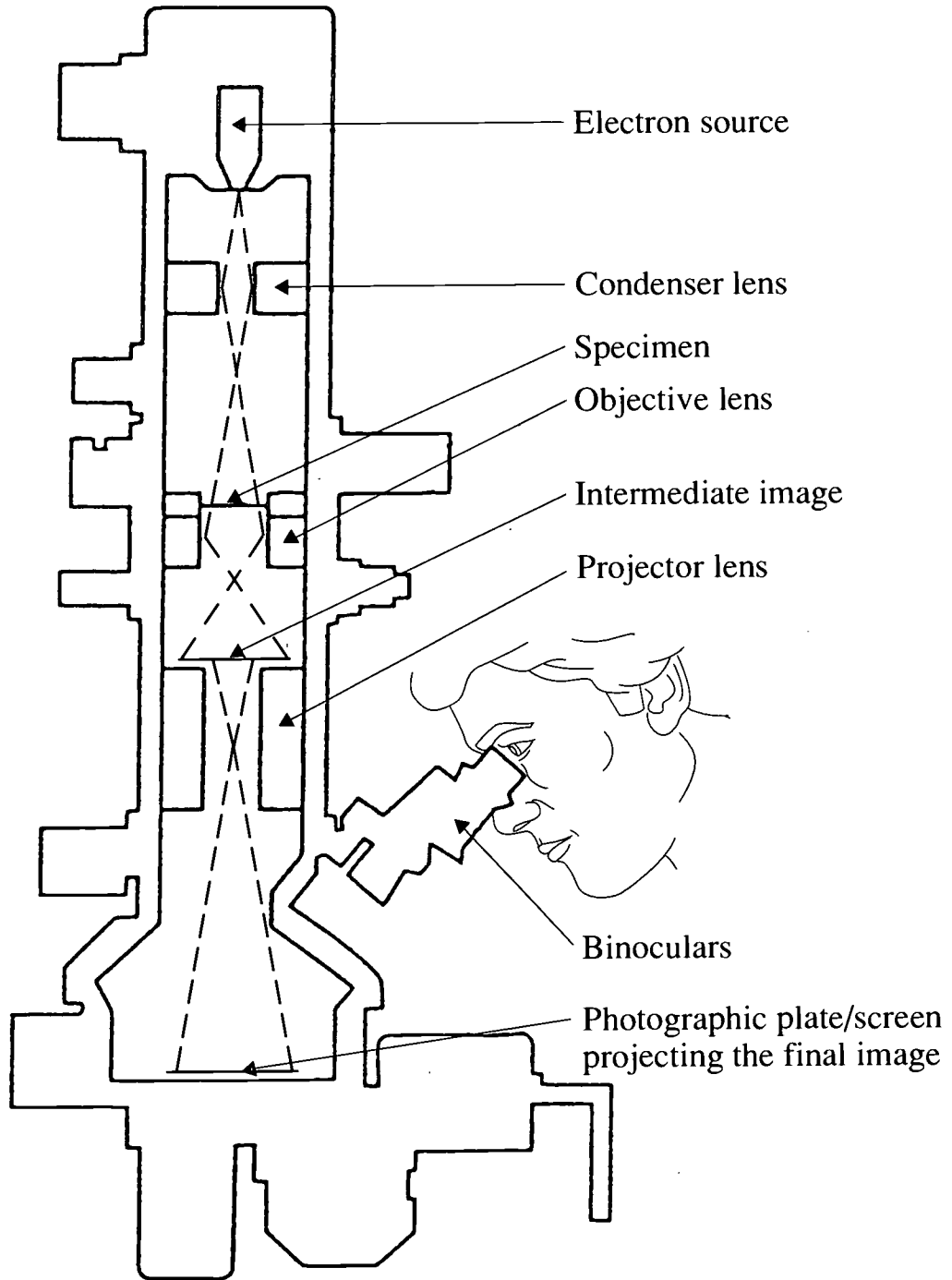


Figure 7-1
Components of the TEM.

A camera system allows the TEM image to be photographed or digitized, stored, and printed.

Electron movement through gases (air) is limited in velocity, so TEM employs a vacuum in its column tube to allow electrons to reach high speeds.

The image recording system consists of a camera-like device mounted below the viewing screen. The camera system allows the operator to record an image on film, utilizing a fine-grain, blue-sensitive emulsion, in 3 x 4 inches (or 6 cm x 9 cm) format. The newest microscopes also allow images to be digitized, stored on magnetic media, and outputted to a printer.

Since molecules in a gas at atmospheric pressure collide with and limit the distance the electron beam can travel, the column tube in the TEM is maintained under a high vacuum. This vacuum, usually in the range of 10^{-5} to 10^{-6} torr (mm of Hg), is achieved by pumping the column with an oil diffusion pump backed by a rotary pump. To limit the column from returning to atmospheric pressure each time specimens are exchanged or film is removed, the microscope is equipped with air locks located at appropriate places along the column. As a result, column vacuum is maintained and specimen exchange is accomplished quickly.

The foregoing description of the TEM is a generalized account, and the reader should be aware that many variations in design exist. Each manufacturer strives to offer an instrument with unique capabilities. Microscopes are marketed in several price ranges, from lower priced, student-level microscopes to very expensive research-grade instruments. Because of their price tag and their required service contract, these instruments remain rare in precollege science departments.

Preparation of Biological Specimens

Biological specimens for TEM viewing require special preparation (analogous to but more exacting than those used in light microscopy).

Methods used in the preparation of biological specimens for TEM are analogous to procedures used for light microscopy. Typically, tissue specimens are chemically fixed, usually using glutaraldehyde and osmium tetroxide as fixatives, then dehydrated with either ethanol or acetone. After thorough dehydration, specimens are infiltrated with, and embedded in, an epoxy resin. The resin is a viscous liquid at room temperature but, when heated to about 60°C for 24-48 hours, it polymerizes to a hard plastic. These blocks are trimmed down to a small face (less than 1.0 mm) and sectioned on an ultramicrotome. A skilled operator can produce a ribbon of sections having a thickness ranging from 50 nm to about 90 nm. The sections are mounted on a metal mesh grid and then stained with heavy metals (such as uranyl acetate and lead citrate) prior to examination with TEM.

Intact organisms are viewed using negative staining techniques to reveal fine detail. Viewing of live specimens is not possible with either the TEM or SEM.

While tissue specimens require embedding and sectioning, particulate specimens, such as viruses, may be examined intact using the negative staining technique. In this procedure, a mesh grid is covered with an ultrathin carbon film which serves as a transparent specimen support. The specimen, such as virus suspension, is mixed with a heavy metal stain such as phosphotungstic acid and deposited on the carbon-coated grid. When viewed in the microscope, the specimen appears light on a dark background, hence the term *negative staining*. This technique is particularly useful for revealing the fine detail of viruses, bacterial flagella, isolated cell components, and macromolecules.

Using the shadow-cast technique, surface topography of the specimen is revealed as a result of shadows cast by evaporated metals.

Another procedure, the *shadow-cast technique*, was one of the earliest techniques developed in electron microscopy. It has been applied to the same types of particulate specimens as described above. In shadow-casting, the specimen is deposited on a carbon-coated grid and then placed in a vacuum evaporator. Under vacuum, a thin film of metal, such as Pt/Pd, is evaporated onto the surface of the specimen at a fixed angle. The metal accumulates on the sides of the specimen exposed to the evaporating source, while the other side of the specimen is shielded from metal deposition. When viewed in the microscope, the surface topography of the specimen is revealed as a result of the "shadows" cast by the evaporated metal.

An extension of the shadow-casting procedure is the replica technique, wherein the surface of an electron-opaque specimen can be examined in the TEM. The specimen is first shadowed, as described above, and then a thin film of carbon is evaporated uniformly onto the surface to form a replica. The specimen itself is chemically dissolved away and the carbon replica is then mounted on a grid for examination. While the replica technique has limited applications in biology, most ultrastructural studies of surfaces are now being done by scanning electron microscopy (SEM).

Advanced Techniques

In recent years, sophisticated new techniques have been developed in biological electron microscopy which have expanded the usefulness of the TEM. The following brief description of advanced techniques is intended to highlight a few of the more well-known ones.

The freeze-etching technique is a method that allows us

Freeze-etching techniques allow viewing of cellular membranes and organelles.

to view the surfaces of membranes and organelles in the TEM, a perspective not possible with thin-sectioning techniques. The procedure consists of four basic steps. First, the specimen is quick-frozen to the temperature of liquid nitrogen (-195° C). Next, the specimen is placed in a vacuum chamber where it is fractured with a cold knife. The knife temperature is held at -195° C and the specimen temperature at -100° C. At high enough vacuum, sublimation of ice will occur (the knife acts as a cold finger) and cell structures will stand out in relief from the receding ice background. This is the etching step. The final step is the production of a shadowed replica of the fractured, etched surface.

EM autoradiography allows us to study the synthesis and movement of cellular substances at the ultrastructural level.

Electron-microscopic (EM) autoradiography is a technique for studying the synthesis and movement of cellular substances at the ultrastructural level. In order to locate the site of synthesis of a particular substance, a radioactive label, for example, tritium, is attached to a known precursor of the substance in question. The labeled chemical is then injected into the animal or supplied to the plant. Sufficient time is allowed to elapse for the label to reach the tissue of interest and to become a part of the synthetic pathway. The tissue is then removed, fixed, embedded, and sectioned.

In a darkroom, a thin layer of photographic emulsion is applied to the sections in such a way as to produce a monolayer of silver halide crystals in contact with the sectioned tissue. The sections are placed in a light-tight box, and several weeks are allowed for exposure of the photographic emulsion. During exposure, the low-energy beta emissions of the radioactive label will expose the silver halide crystals that are located directly above the radioactive source. When the emulsion is developed, the location of the labeled substance is accurately revealed and the relationship of that substance to cell organelles can be determined.

EM immunocytochemistry allows us to view ultrastructural localization of antigenic material.

Electron microscopic immunocytochemistry is a technique that has become very popular in recent years. The procedure is based on the specificity of the antigen-antibody reaction, and it permits ultrastructural localization of antigenic components in tissue sections or whole mounts using electron-opaque markers. In practice, an antigen (usually an isolated, purified protein or complex carbohydrate) is injected into an animal (often a rabbit) in order to produce a specific antibody. After several weeks, a sample of blood is taken from the rabbit and the

antibody-containing fraction is isolated and purified. Next, ultrathin sections of the tissue being studied are incubated briefly with the antibody fraction. Hopefully, the antibodies will bind specifically to the tissue antigens.

The antibodies are then marked with an electron-opaque tag. One of the most widely used ultrastructural markers is colloidal gold conjugated to Protein A. Microscopic beads of colloidal gold are commercially available in a variety of sizes, with 5, 10, 15, and 20 nm being the most common. These particles are very electron-opaque and are uniform in size and shape. Protein A is a bacterial protein that has the unique ability to bind to the Fc portion of the antibody molecule. When the tissue sections are viewed in the electron microscope, the ultrastructural location of the antigen is revealed by the presence of the gold marker. The foregoing discussion describes one commonly used technique in immunocytochemistry. The reader should be aware that a wide variety of techniques and variations has been developed and that entire books have been written on this subject.

Energy dispersive X-ray analysis allows for elemental analysis of thin-sectioned tissue.

A Dewar flask is a double-walled vessel, with an internal vacuum used to store liquid gases temporarily.

Computerized image analysis allows for storing image for later viewing and reconstruction.

Morphometry is the measurement of external form.

Energy dispersive X-ray analysis is an analytical tool requiring some expensive instrumentation. This includes a semiconductor X-ray detector that is mounted in the microscope column near the specimen, a liquid nitrogen Dewar flask to maintain the detector at low temperature, electronic equipment to process the signals, and a computer to analyze the data. With these components, elemental analysis of thin-sectioned tissue can be performed. The technique has its limitations, but essentially the operator is able to determine most of the elements present in a sample, both qualitatively and semi-quantitatively, if they are present in detectable quantities. The system functions by analyzing the energy of characteristic X-rays emitted by the specimen when it is bombarded by the electron beam. The computer displays the data as a spectrum, with each peak identifiable as a particular element by its characteristic energy.

One of the most recent developments in EM is computerized image analysis. Methods are now available to digitize images and store them on magnetic or optical media. These images can then be recalled, electronically processed and enhanced, and subjected to computerized morphometric analysis. With the appropriate program, ordinary desktop computers can also be used to generate three-dimensional reconstructions from

two-dimensional images. Computer techniques appear to have a bright future in electron microscopy.

Another new and expensive type of instrument is the scanning transmission electron microscope. The STEM combines many of the features of TEM and SEM into one instrument. Using a combination of signal detectors, the STEM is capable of providing several imaging modes. It can produce both TEM and SEM images and can be coupled with an X-ray detector for elemental analysis of relatively thick sections. These microscopes can be configured in several ways, with the more elaborate versions being referred to as analytical electron microscopes. This discussion of electron microscopy has been necessarily superficial. To cover the subject adequately would require an entire textbook. In fact, volumes have been written on just such subjects as microscope operation, fixation of biological specimens, ultramicrotomy, positive staining, immunocytochemistry, and X-ray analysis. For the reader who would like more information on the general subject of transmission electron microscopy, a suggested reading list for this chapter follows.

Questions Most Commonly Asked by Students

Question 1: Can the TEM produce pictures in color?

No. Color is a function of visible light. Electron microscopes use an electron beam, not light, to image the specimen. However, a modern electron microscope system can produce artificial (computer-generated pseudocolor) color images. Usually color is added to enhance interpretation.

Question 2: If an electron beam is invisible, how do we see an image on the TEM viewing screen?

The viewing screen is coated with a phosphorescent material that, when struck by electrons, emits a greenish-yellow light.

Question 3: Can you think of any familiar, everyday device that is similar in its functional principle to the electron gun?

Yes. The light bulb

Question 4: In order for the TEM to produce usable images of biological specimens, the tissue must be cut into extremely thin sections. Why?

In the TEM, as in the light microscope, the imaging beam must pass through the specimen. An electron beam does not possess sufficient energy to pass through a whole mount, or even a thick section. Consequently, ultrathin sections (on the order of 50-90 nm) are necessary.

Appendix A

(for figures on following pages)

Plate 1 - Figure 1 — The Hitachi H-600 transmission electron microscope:

- 1 - electron gun
- 2 - condensor lenses
- 3 - objective lens
- 4 - projector lenses
- 5 - viewing chamber
- 6 - specimen exchange port.

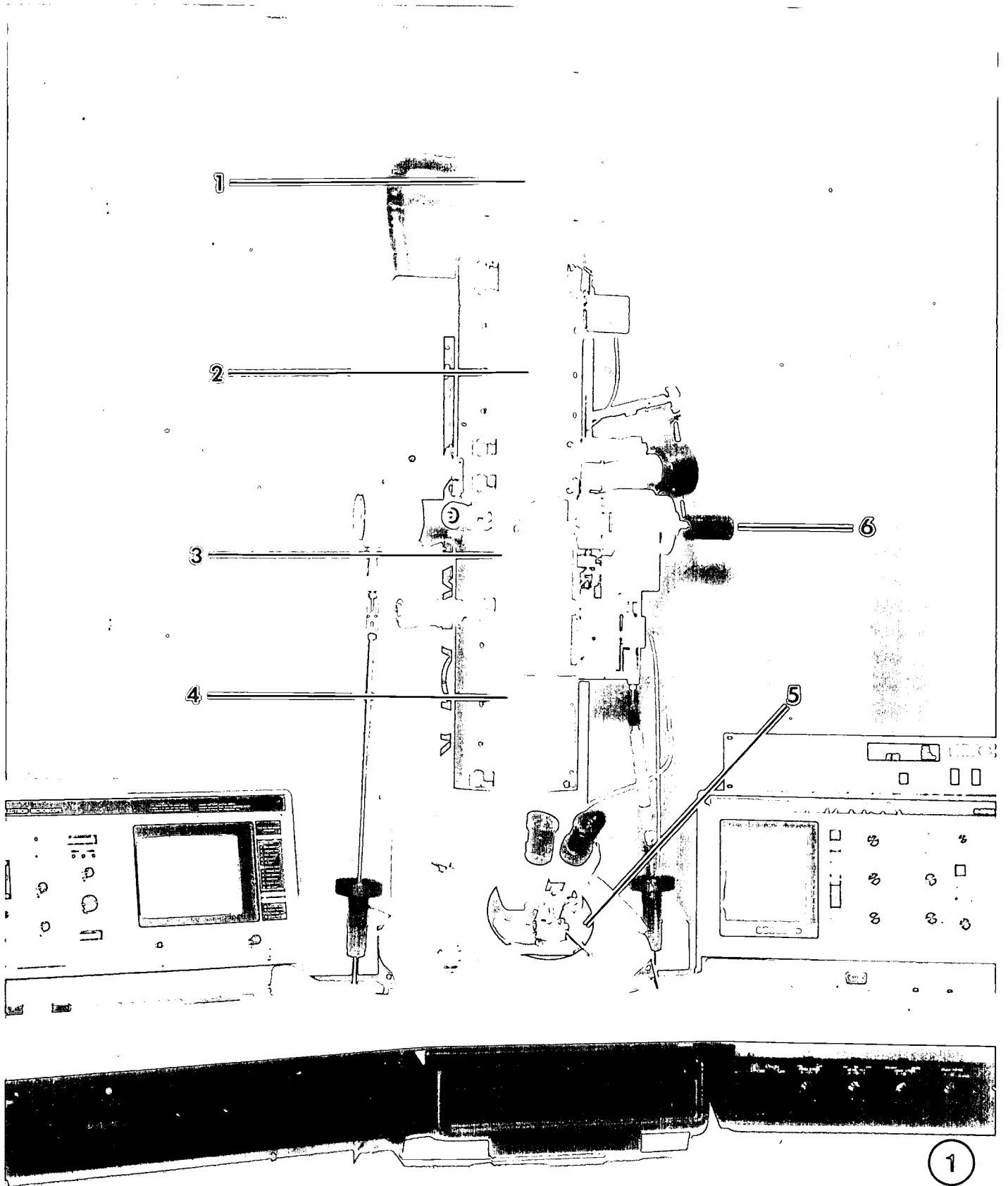
Plate 2 — (Illustrated examples of thin-sectioned specimens)

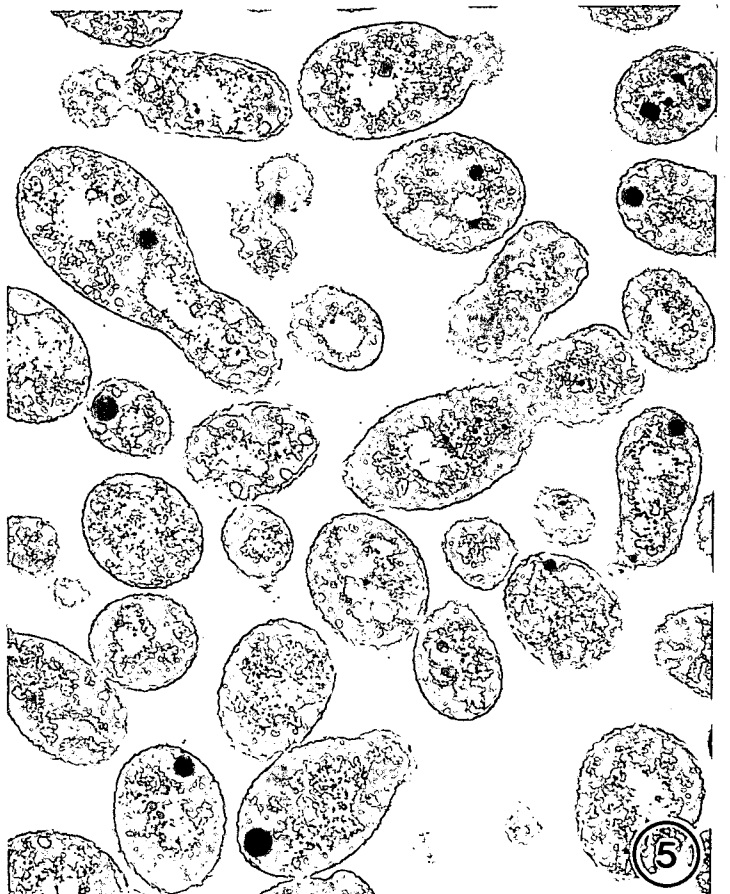
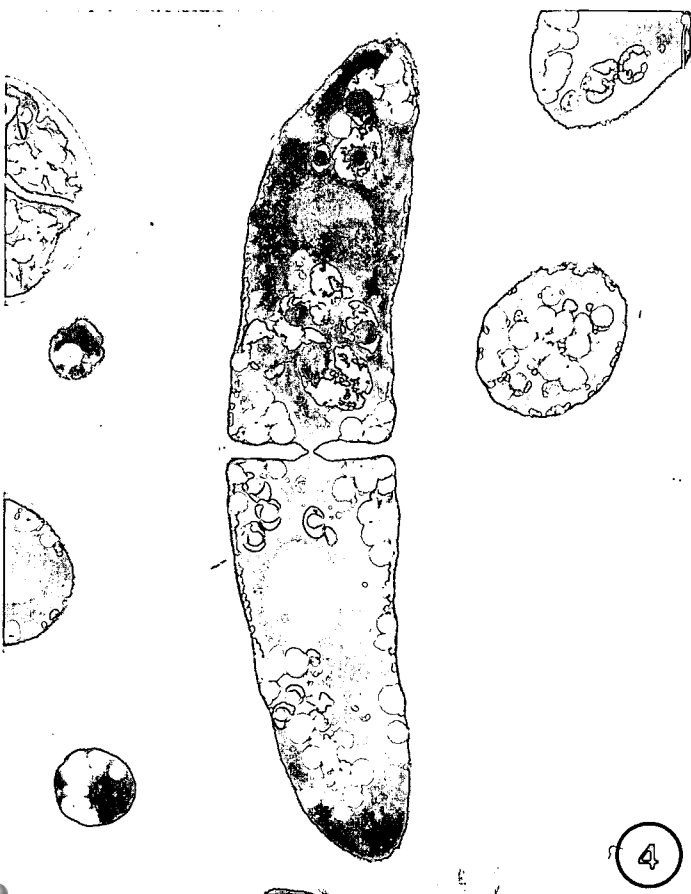
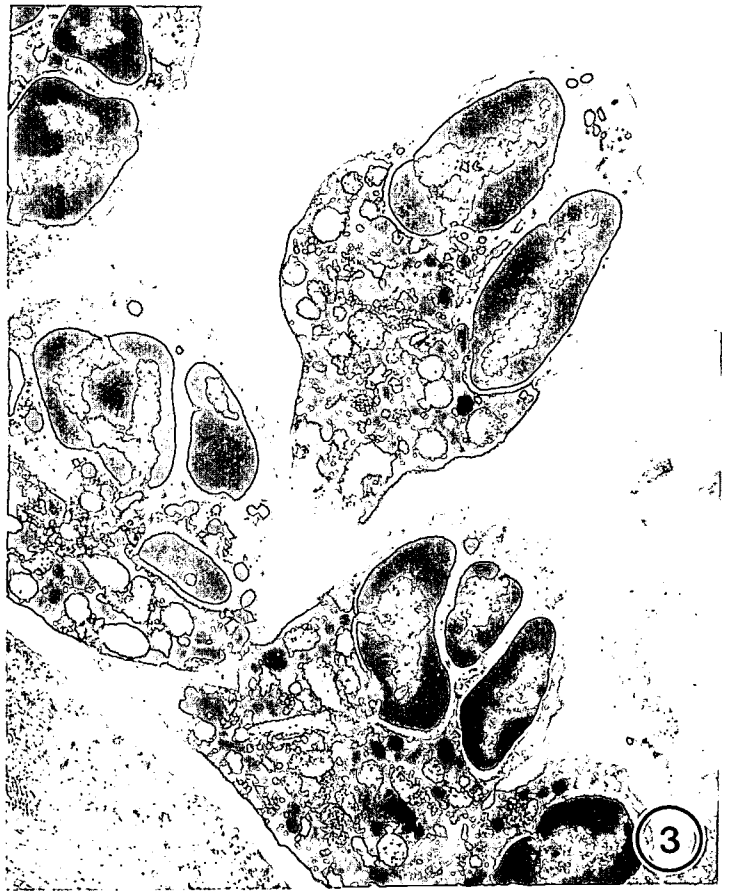
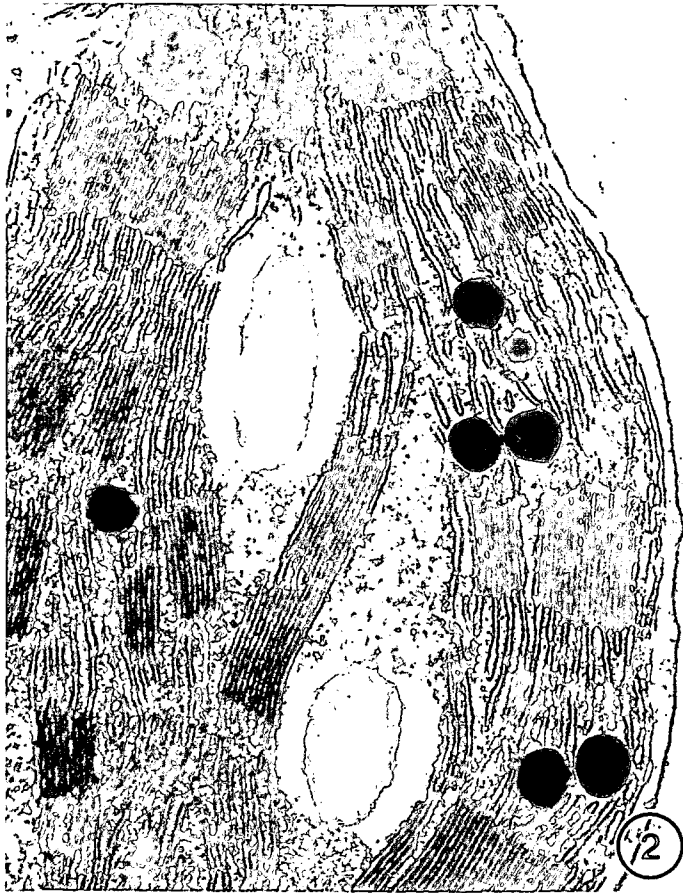
Figure 2 - A portion of a chloroplast from a tobacco leaf (x 31,500)

Figure 3 - Rabbit leukocytes (x 7,500)

Figure 4 - Conidia of the fungus *Fusarium solani* (x 6,500)

Figure 5 - *Agrobacterium tumefaciens* (x 15,500)







Dr. J.A. White introduces Mike George to the JEOL 100B TEM. Mike is a science student at West Junior High School in Columbia, MO. (The TEM is manufactured by JEOL, Inc. of Peabody, MA.)

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Bozzola, J.J. & Russell, L. D. (1992). *Electron microscopy: Principles and techniques for biologists*. Boston: Jones and Bartlett, Publishers.

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Dr. M.F. Brown is Professor Emeritus in the Department of Plant Pathology at the University of Missouri-Columbia. He was manager of the Electron Microscope Facility from 1968-1992. His areas of specialization are electron microscopy, mycology, and forest pathology.

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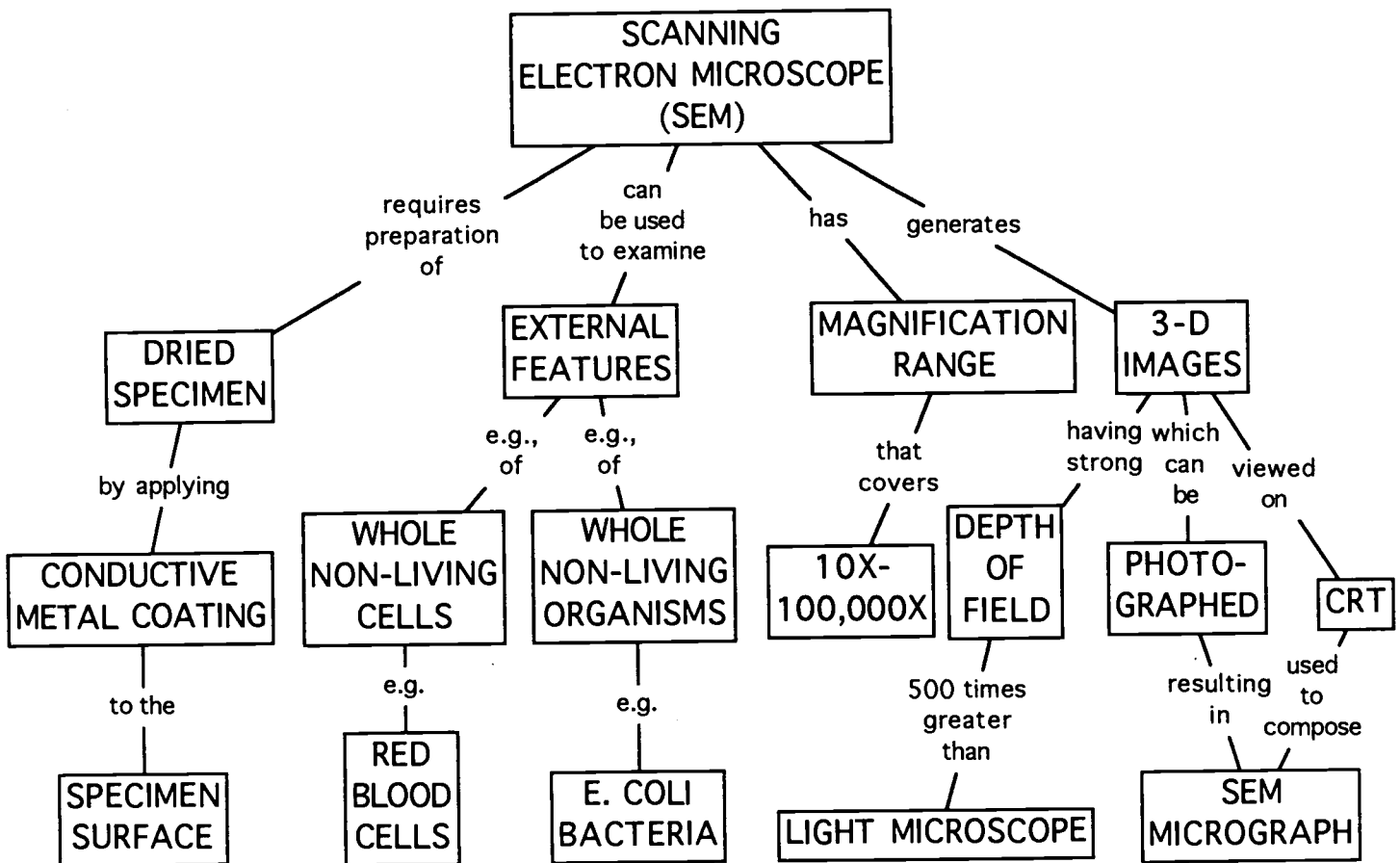
SCANNING ELECTRON MICROSCOPY

8

Merton F. Brown and Jerry A. White

Overview

1. The basic scanning electron microscope (SEM)
2. Principles of operation of the SEM
3. Generation of the SEM image
4. Preparation of the specimens for SEM viewing
5. Improvements in SEM technology



Scanning Electron Microscopy

An SEM can be used for observation of an entire specimen.

The SEM produces a three-dimensional image at a depth of field 500 times greater than light microscopy (at the same magnification).

Although transmission electron microscopes (TEM) are capable of high resolution and high magnification, they are limited to the two-dimensional images yielded by ultrathin sections. In order to examine whole specimens, one must employ the scanning electron microscope (SEM). The great strength of the SEM lies in its ability to produce three-dimensional images having great depth of field. The SEM has a depth of field approximately 500 times that of the light microscope at the same magnification. When great depth of field is coupled with high resolution (4-5 nm) and a broad range of magnification (10x-100,000x), the result is an instrument with unique capabilities.

The theoretical concepts that form the basis for the SEM date back to the 1930. However, it was not until 1965 that the Cambridge Instrument Company introduced the first commercially successful SEM. Since then, development has been rapid, with greatly enhanced instrument performance and a broad range of capabilities.

The Basic Scanning Electron Microscope

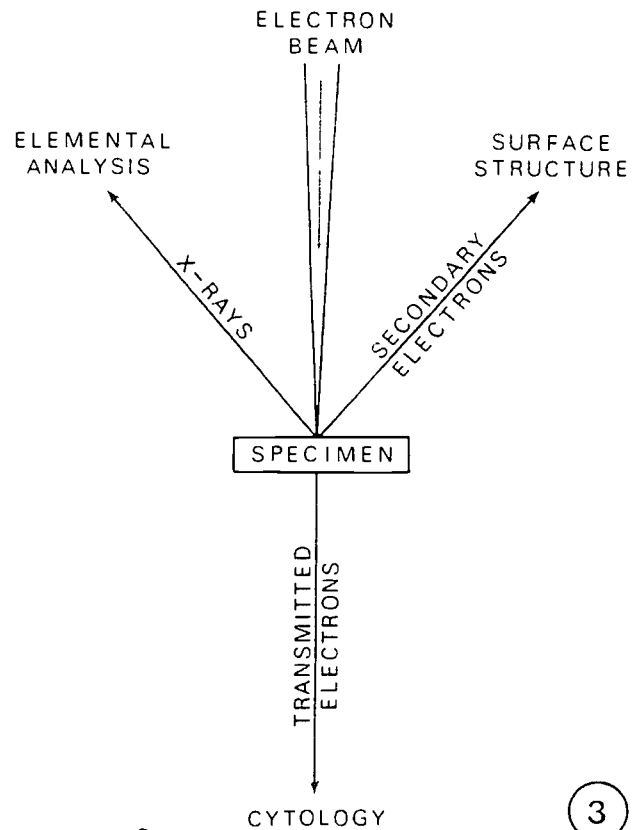
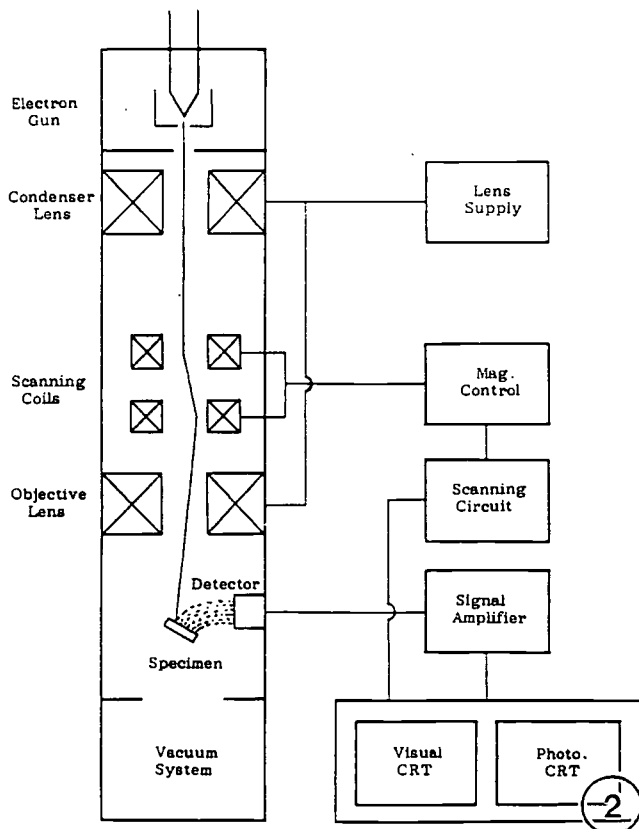
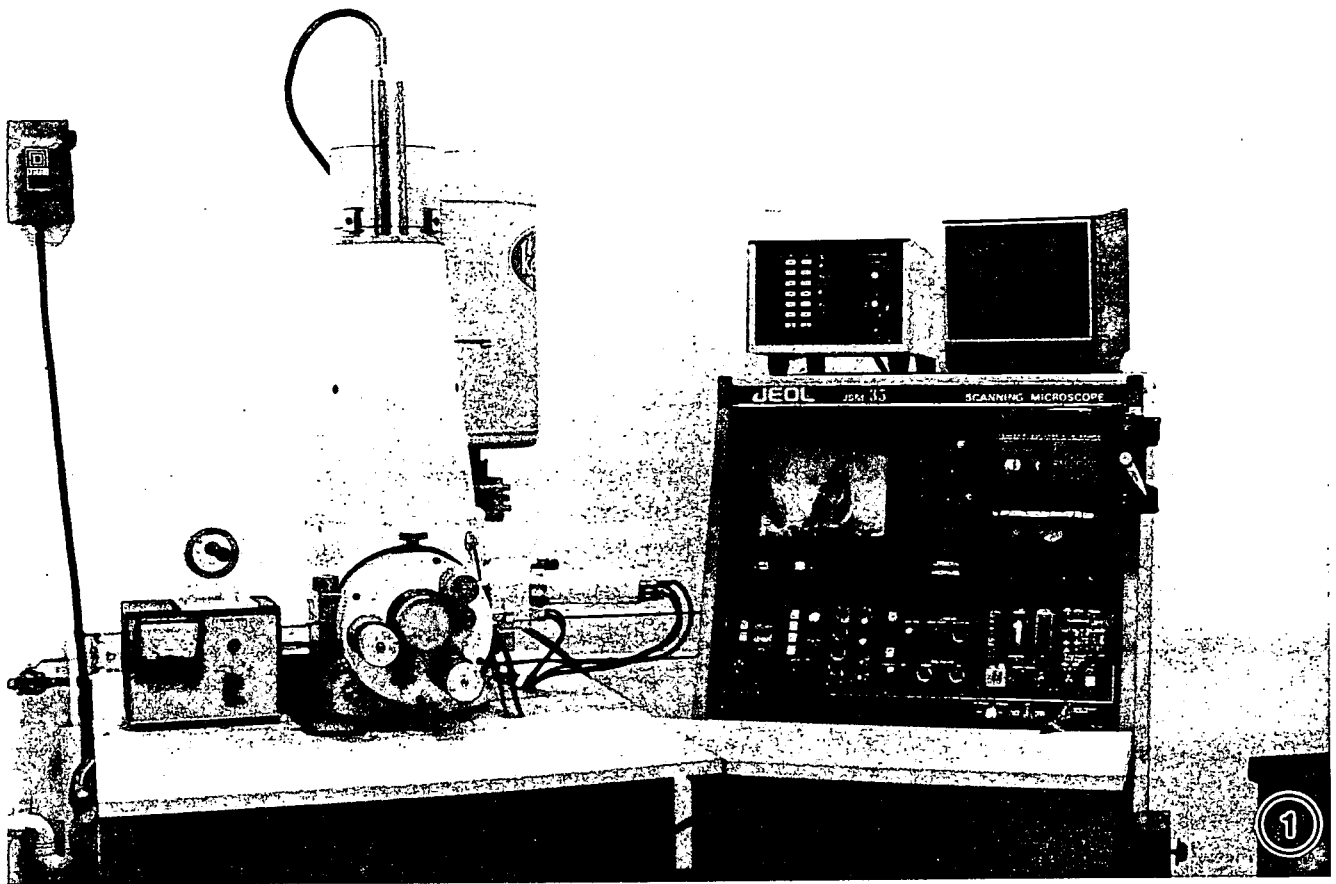
The basic SEM is composed of six systems: (1) the illumination system, (2) the lens system, (3) the signal generating system, (4) the signal detecting system, (5) the image recording system, and (6) the vacuum system. (See the following page for a photograph: #1 of the SEM, and two line drawings – #'s 2 & 3 – of the SEM principle.) The illumination system (the electron gun) and the vacuum system are basically the same as those used in the TEM (see Chapter 7).

The lens system of the SEM consists of a condenser lens and an objective lens. The function of these lenses, however, is quite different from that in the TEM. Rather than form a magnified image of the specimen, these lenses serve to form the electron beam into a finely focused probe of minimum diameter. While the diameter of the electron beam at its source (the tungsten filament) may be as large as 5 m, the diameter of the beam at the surface of the specimen may be 5 nm or less. Minimum beam diameter is critically important because attainable resolution can be no better than the diameter of the beam striking the specimen surface.

Electron beam diameter influences the resolution of the SEM image.

The electron beam scans the surface of the specimen to produce a detailed image.

Signal generation in the SEM involves "scanning" the electron beam across the surface of the specimen. Scanning coils (horizontal and vertical) move the beam left to right and top to



As the electron beam scans the surface of the specimen, it collides with surface electrons, exciting them, and causing secondary electrons to be emitted.

bottom in a raster pattern of closely spaced parallel lines. When the beam strikes the specimen, it penetrates the surface, causing excitation of the atomic structure. This excitation, caused by the collision of beam electrons with specimen atoms, results in the emission of several different types of radiation. Of greatest interest to biologists are the low energy, secondary electrons. Secondary electrons are emitted only from a very thin layer of the specimen's surface. Consequently, they are useful for generating images of the fine structure of the surface of the specimen.

When secondary electrons are emitted from the specimen surface, they are attracted to an electron detector. This detector is located above and to one side of the specimen. The secondary electrons travel in a curved path toward the detector, attracted by its positively charged surface. Each secondary electron that strikes the detector is converted to a light pulse by a scintillator and then fed to a photomultiplier. The photomultiplier produces a photocurrent which is amplified and then used to control the brightness on a cathode ray tube (CRT).

The emitted secondary electrons are detected, converted to light, and processed by the photomultiplier and CRT circuitry for the generation of visible image.

Since the scanning coils in the microscope and the deflection coils in the CRT are synchronized, variations in secondary electron emission from the specimen are seen on the CRT screen as corresponding variations in brightness. Each image point on the CRT corresponds to an image point on the specimen. As the beam scans the specimen surface, a detailed image is formed on the CRT in much the same way that a television image is formed. The specimen can be moved along the X and Y axes, as well as tilted and rotated. In this way, all aspects of the specimen can be viewed and, if desired, photographed.

As the length of the scan line is decreased, magnification of the SEM image is increased.

Since there are no magnifying lenses in the SEM, how is image magnification determined? Very simply, it is the ratio of the length of the scan line on the viewing CRT to the length of the scan line on the specimen. For example, if the length of the scan line on the specimen is 10 mm, and the image area on the CRT is 10 cm wide, then the image magnification is 10X. If the length of the specimen scan line is reduced to 5 mm, then the image magnification becomes 20X. In this way, a stepwise reduction in the size of the area scanned on the specimen results in a stepwise increase in magnification.

Photography in the SEM is simple and straight forward. There are two CRTs — a viewing CRT and a photographic CRT. The viewing CRT is a long persistence CRT designed for visual

Images from the SEM appear to have diffuse illumination.

The SEM permits high magnification and resolution, while producing three-dimensional images.

To assure quality SEM images, the specimen needs to be water-free (dry) and treated to remain electrically conductive.

During the removal of water from the specimen, surface tension increases, which distorts the actual surface of the specimen.

observation by the microscopist. In order to photograph the image, a short-persistence, high resolution CRT is used. The photographic CRT is usually fitted with a Polaroid film back using 4x5 positive/negative film. Because of the way in which the secondary electron image is formed, the SEM photographically records the specimen as though it were illuminated by a diffuse but directional light source. Since most objects that are observed in nature are lighted in a similar way, SEM micrographs look realistic and require virtually no conscious effort for interpretation of depth and dimension. The combination of high magnification, high resolution, and three-dimensional portrayal has made the SEM an invaluable tool for biological research.

Preparation of Biological Specimens for the SEM

It would be tempting to offer the reader a single, standard *recipe* by which most biological specimens could be properly prepared for SEM study. However, the goal of specimen preparation is to achieve lifelike, artifact-free preservation, and any attempt to use a universal approach will generally result in disappointment. The need for lifelike preservation means that each new specimen represents a new challenge to the investigator.

Typically, biologists are confronted with two major problems that must be resolved if high quality SEM micrographs are to be obtained. First, since the specimen is ultimately subjected to a vacuum of 10^{-5} to 10^{-6} torr, and is scanned with a high energy electron beam. All free and bound water must be removed from the specimen in a way that will minimize alterations in specimen morphology. The second problem is that most biological materials are composed of light elements that, when dry, are poor electrical conductors. In order to obtain acceptable SEM images, it is necessary to treat the specimen in such a way as to render it electrically conductive, while maintaining lifelike structure. In summary, the goal of specimen preparation is to produce dry specimens which faithfully represent the morphology of the living organism and are uniformly conductive.

Air-drying is obviously the simplest way to remove water from a fresh specimen. Unfortunately, during air drying, tremendous surface tension forces (as much as $40,000 \text{ kg/cm}^2$) are exerted on the specimen surface. Such forces will readily collapse the surface of most soft specimens. Clearly, techniques must be used that bypass the surface tension phenomenon associated with liquid-air interfaces.

Critical-point drying uses chemical fixation and liquid carbon dioxide in the drying process to avoid changes in the morphology of the specimen.

The most widely used drying procedure is critical-point drying (CPD). This method requires chemical fixation (using glutaraldehyde/osmium tetroxide), dehydration in an organic solvent (e.g., ethanol), and then drying liquid carbon dioxide (CO₂) in a critical-point apparatus. The technique is based on the physical principle of critical point — the combination of critical temperature and critical pressure. When an appropriate liquid (e.g., liquid CO₂) is placed in a sealed container and heated to its critical point, all liquid is instantaneously converted to a supersaturated gas. If a specimen is in the liquid CO₂ when it reaches its critical point, no phase boundary will pass through the specimen, there will be no liquid-gas surface tension, and the specimen will be dry. In essence, the CPD avoids the gross morphological damage associated with air-drying or drying from volatile solvents. Critical-point driers are small, moderately priced, and a standard fixture in SEM laboratories.

Once the specimen is dried, it is mounted and given a conductive coating.

After the specimen has been dried, it is mounted on an aluminum support stub. The sample is attached to the stub with a conductive tape or glue in order to provide a continuous electrical path from the specimen to the microscope stage. The final step is to provide the specimen with a conductive metal coating. This is usually accomplished in a sputter-coater, an instrument that deposits an ultrafine layer of gold or gold/palladium alloy (10-30 nm thick) uniformly over the surface of the specimen. The sputtercoater is based on the technique of low-vacuum plasma sputtering, a common thin-film coating technology widely used in industry. After coating, the sample is ready to view with the SEM.

Some Improvements in Instrumentation and Technique

Examples of some recent technological improvements include X-ray analysis, immunolabeling, stereomicroscopy, ultrahigh resolution SEM, scanning transmission electron microscopy (STEM), and a variety of exotic specimen preparation procedures. X-ray analysis and immunolabeling are used in basically the same way as in the TEM. (See Chapter 7 for discussion of those techniques.)

Stereomicroscopy is used to create a three-dimensional image to aid in interpreting structural uncertainties on the specimen.

Stereomicroscopy involves the production of stereo pairs — two micrographs are taken of the same specimen area, with one being tilted slightly (6-8 inches) with respect to the other. The resulting stereo pair is viewed with a stereoscope (which produces an integrated 3-D stereo image). Stereo pairs are useful in interpreting structure and resolving ambiguities.

Ultrahigh SEM uses small beam diameters to obtain high resolution.

Ultrahigh resolution SEM utilizes improvements in electron gun technology which permit a smaller beam diameter (spot size) and improved signal-to-noise ratio. The lanthanum hexaboride gun and the field emission source are examples of such improvements. Resolution in the 1-2 nm range is possible with the latest field emission instruments.

A SEM can be fitted with a transmission electron detector to obtain the benefits of both scanning and transmission electron microscopy.

As mentioned in the chapter on TEM, the STEM combines many of the features of the TEM and SEM in one versatile instrument. However, the STEM tends to be rather expensive. For those SEM users who need the versatility of the STEM mode of operation (e.g., to obtain transmitted electron images from sectioned specimens), there is an alternative to the dedicated STEM. Most SEM manufacturers offer a *transmitted electron detector* as an optional accessory. This detector allows the SEM to function in the STEM mode and yields cytological information from thin sections that is similar to that obtained with the STEM.

Techniques for specimen preparation have been improved to eliminate the necessity of chemical fixation and drying of the specimen.

Improvements in specimen preparation techniques have centered on ways to avoid the use of chemical fixation, dehydration, and drying. Two of the most promising alternatives to critical-point drying involve ultrarapid freezing of specimens. In the first method, freeze drying, the fresh specimen is quick-frozen with liquid nitrogen. The frozen specimen is then placed in a freeze-drying apparatus where the combination of low temperature and vacuum dry the specimen. The freeze-drying technique avoids the use of chemical fixatives and dehydrating solvents and the physical/chemical changes in the specimen associated with their use. However, it is a time-consuming procedure and is not always successful.

Newer specimen preparation techniques include freeze-drying and low-temperature SEM.

The second technique is generally referred to as *low-temperature SEM* or *cryo-SEM*. In this method, the fresh specimen is quick-frozen with liquid nitrogen and then examined in the SEM in a frozen-hydrated state. Low-temperature SEM produces specimens that exhibit superior morphological preservation due to the fact that frozen-hydrated specimens are not subjected to chemical fixation, dehydration, or drying.

Other techniques used for specimen preparation include solution-coating and cryofracture.

Solution-coating techniques and cryofracture techniques are two examples of improvements in specimen preparation that do not require special equipment. Solution-coating involves impregnating the specimen with heavy metals in solution. One such procedure, known as OTO, utilizes a solution of thiocarbonyl-drazide to bind additional osmium tetroxide to the specimen. This results in improved stability and conductivity.

In some cases, OTO can replace sputter-coating and, in others, can enhance it. Cryofracturing is an excellent way of examining internal features of biological specimens. In a typical procedure, known as *ethanol cryofracture*, the specimen is first fixed with glutaraldehyde and osmium tetroxide and then dehydrated in ethanol. While in 100 percent ethanol, the sample is quick-frozen in liquid nitrogen. The frozen specimen is then fractured with a razor blade. The fractured pieces are critical-point, dried, mounted, and sputter-coated. Cryofracturing avoids many of the undesirable characteristics of wet- or dry-sectioning and frequently yields visual information not obtainable with other techniques.

This discussion of scanning electron microscopy is intended only as an introduction to the subject. For those who would like to pursue the subject in more detail, a list of suggested readings follows.

Questions Commonly Asked by Students

Question 1: Can living organisms be examined in the SEM?

Generally speaking, no. A living organism, if placed inside the specimen chamber of the SEM, would instantly be subjected to a lethal environment (e.g., high vacuum, localized heating, drying, and structural collapse).

Question 2: Scanning coils are used in the SEM to sweep the electron beam across the specimen in a series of horizontal and vertical movements called a "raster pattern." What common household devices use a similar electron beam, scanning coils, and raster patterns?

A television set and a computer monitor.

Question 3: Unlike the TEM, the SEM has no magnifying lens. How does the SEM produce a magnified image?

The image is displayed on a television-like CRT having a fixed size (e.g., 15x20 cm). If the area scanned on the specimen is made progressively smaller, then the magnification of the displayed image becomes progressively higher.



Dr. Jerry A. White introduces Mike George to the JEOL 35 SEM. Mike is a science student at West Junior High School in Columbia, MO. (The scanning electron microscope shown is manufactured by JEOL, Inc. of Peabody, ME.)

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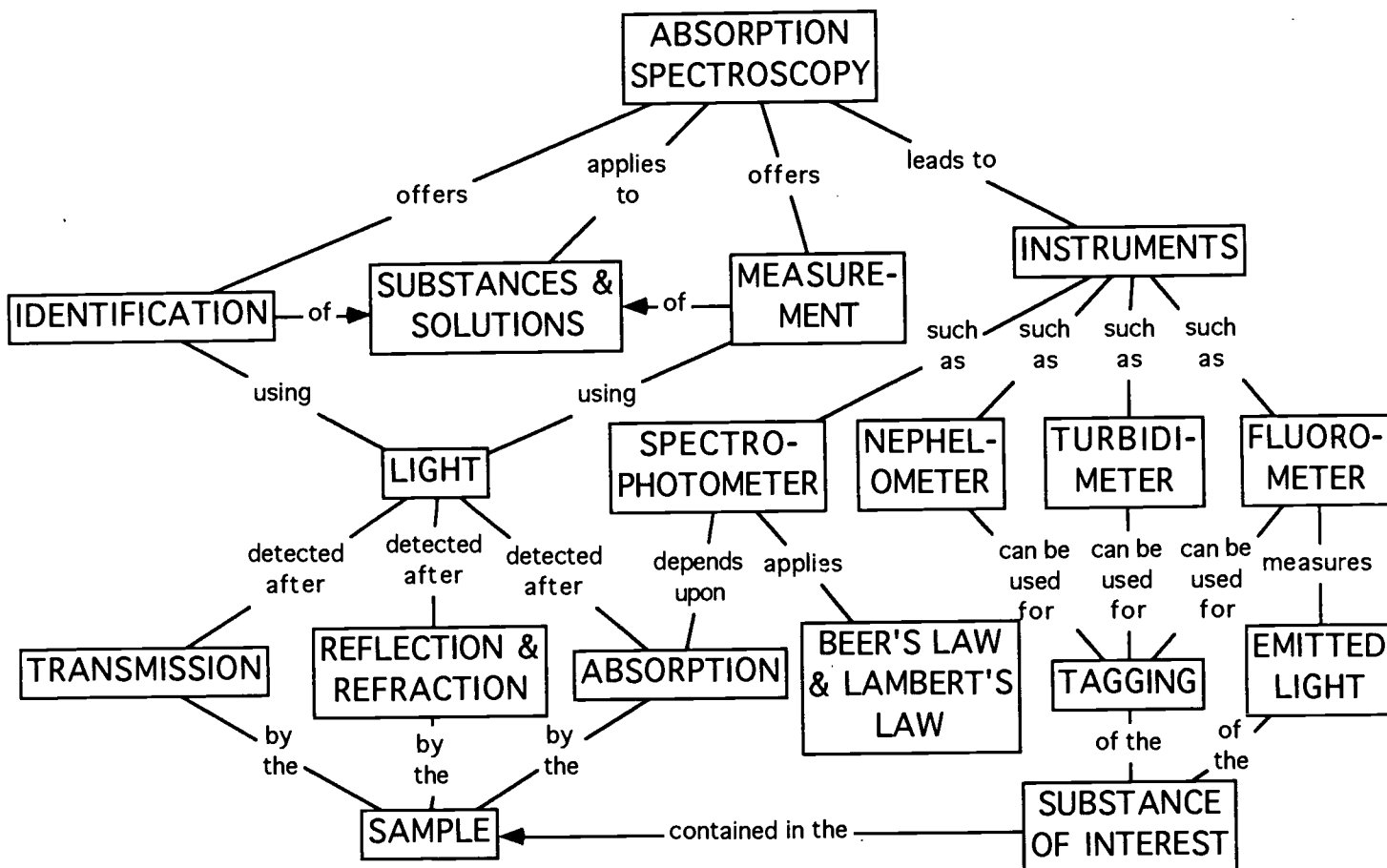
ABSORPTION SPECTROSCOPY

9

John S. Davis

Overview

1. Properties of light
2. Light and matter
3. Light for identification and measurement
4. Spectroscopic instrumentation



Absorption Spectroscopy

Spectrometry uses spectrographic techniques to derive the physical constants of materials.

Spectroscopy is the study and measurement of specific interactions of light with matter. Analysis of the light yield both quantitative and qualitative results. Quantitative determinations are tests that measure the amount of substance present; qualitative determinations reveal the type of substance present. For decades, scientists and medical researchers have utilized the unique properties of light reactions to identify and measure concentration. This chapter will discuss the properties of light and the use of light for specific identification and measurement of unknown substances or solutions. Included is a description of various types of instrumentation and methodologies currently in use by scientists, researchers, and medical professionals.

Properties of Light

Light is a form of radiant energy that has wave-like properties.

Light, by the simplest definition, is a form of radiant energy. Specifically, light is energy in the form of electromagnetic radiation (EMR). Light is usually described in terms of its wave-like properties, which provide a variety of means to describe and differentiate various types of electromagnetic radiation. Properties of light include speed, wavelength, amplitude, and frequency.

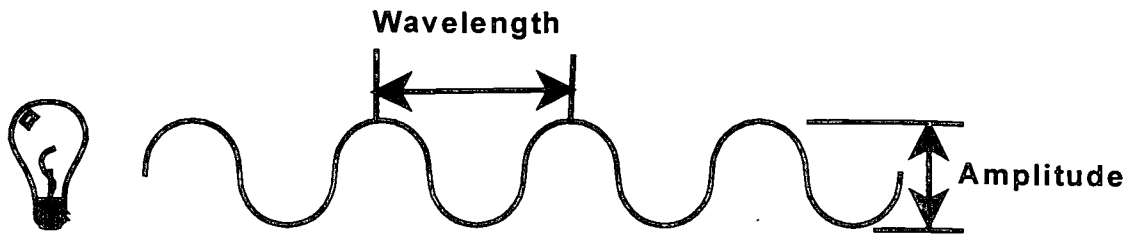
The various media in which the light travels alter the speed of the light.

The speed of light is a constant (3×10^8 meters per second) and is, therefore, not suitable for differentiating light. The speed does change, however, with respect to the medium the light is traveling through. Wavelength, amplitude, and frequency *do* provide differentiating properties of various types of light. When describing light, wavelength refers to the distance between peaks or troughs of a continuous wave. *Wavelength* is abbreviated with the Greek letter lambda (λ) and is measured in units called *nanometers* (nm) or 10^{-9} meters. Wavelength is the distance from one crest to the next crest on a wave. The *amplitude* of the wave is the maximum displacement of a wave with respect to its position. *Frequency* is the number of waves that pass a given point per second as the light wave travels past that point (see Figure 9-1).

Amplitude is the maximum absolute value (energy) attained by the wave.

Light can be differentiated by its wavelength, amplitude, and frequency.

Wavelength, amplitude and frequency all vary with types of different light and provide suitable means for differentiating light. Wavelength has become the accepted method for identifying light in the biological and medical laboratory. Although light varies by energy too, this is not used for light differentiation, but it can be used for identification and quantification. As



Long Wavelength = Low Frequency



Short Wavelength = High Frequency

Figure 9-1

A comparison of wavelengths of visible light.

the wavelength decreases, the energy contained in the wave increases. The characteristics of wavelength are used to describe various types of light. As wavelength varies, changes occur in the light's frequency and amplitude, as well as its color. Therefore, color can be used to identify various light sources.

The combination of all wavelengths of visible light is known as white light.

White (polychromatic) light can be separated into its simple colors (monochromatic light) ranging from violet to red — the visible spectrum.

Light generated by the sun or household lamps is described as *white light*, which is a combination of all wavelengths or colors of light in the visible light range. Visible light, however, represents only a small portion of the electromagnetic radiation spectrum (see Figure 9-2). Light that is known as the *visible range* is light with wavelengths of 380 nm to 750 nm. *Violet light* is found at one end of the visible light range and has the shortest wavelength the human eye can see. As the wavelength increases, light changes in color as follows: blue, green, yellow, orange, and red. Wavelengths shorter than violet (380 nm) or longer than red (750 nm) cannot be seen by the human eye.

Light and Matter

When light strikes a substance, it can be transmitted, absorbed, reflected, or refracted.

Light travels in a straight line. When light strikes matter, a number of reactions can take place. Transmission occurs when light striking a substance enters the substance and travels through it. An example of the transmission phenomenon is sunlight passing through a windowpane. *Absorption* occurs when light enters a substance and some or all of its radiant energy is taken up by the substance. For example, partial absorption occurs when sunlight passes through sunglasses. *Reflection* results when light is returned by the surface of a substance, as when a mirror reflects light. *Refraction* occurs when light is allowed to enter a substance or medium and pass through it, but the light path deviates from the original straight line. Refraction is observed when straight objects placed in a glass of water appear to be bent.

Substances absorb certain wavelengths of light and transmit others.

The concept of absorption is central to utilizing light to identify and quantify. To realize how absorption is useful, some understanding of chemistry is necessary. Matter is composed of protons, neutrons, and electrons. Electrons exist in a variety of energy states. To change energy levels from a ground state to an excited state requires adding a very specific amount of energy. This energy is specified by the unique molecular structure of the substance and orbital electrostatic forces. That is, only an exact energy amount or wavelength of light will be ab-

Adapted figure--used by permission of W.B. Saunders Company. Adapted from Clinical Laboratory Instrumentation and Automation Principles, Applications, and Selection by Ward, Lehmann, and Leiken, 1994, Fig. 2-1, p. 42, (ISBN 0-7216-4218-7)).

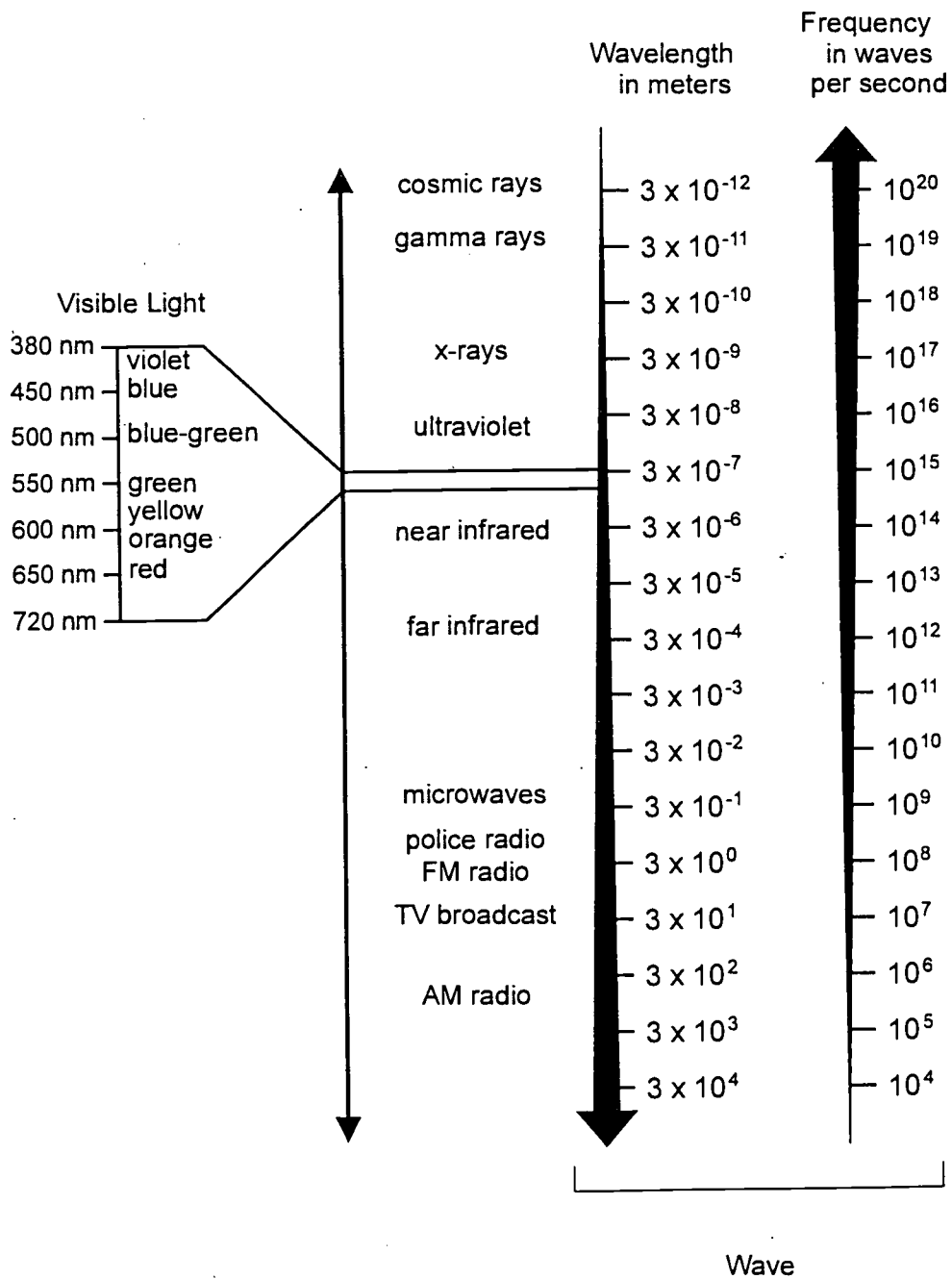


Figure 9-2
Electromagnetic spectrum.

sorbed and excite the molecule. As energy varies inversely with wavelength, specific substances will absorb specific energy units (wavelengths) for excitation. Wavelengths or energies that do not correspond to a substance's specific energy states will be transmitted, reflected, or refracted.

Light for Identification and Measurement

Light is a wave-like form of energy that reacts with matter. This explains why objects appear to be a certain color to the eye. To further explore this phenomenon, imagine a glass of juice is placed on the counter. How would you initially identify the type of juice in the glass? By its color, of course. Why does orange juice appear orange and not purple? This question can be answered using the molecular absorption/ transmission phenomena discussed in the previous section.

Orange juice appears orange because it is molecularly different from any other juice. Orange juice's particular molecular structure absorbs different specific energies or wavelengths while allowing others to be transmitted. Remembering that all visible wavelengths are needed to produce white light and, if the juice absorbs some of the light (wavelengths) that is illuminating it, then the light transmitted cannot appear white—the juice will have a specific color. The juice appears orange because the unique chemical structure of the orange juice *absorbs* specific wavelengths of light from about 480 nm to 490 nm which represents greenish-blue light. All other light is transmitted, which combines to form a color perceived by your eye, and this color has been defined as orange.

White light with an absence of greenish-blue light is orange. The color a solution or object appears is due to the absence of the wavelengths that are absorbed by the unique molecular structure of that solution or object. In the visible light range, there are corresponding light wavelengths that are absorbed to create the color of the solution or object. The remaining color the human eye detects is called the *observed* or *complementary color* (see Table 9-1).

Identification of a substance, such as juice, can be made using the color observed. Determining how much of the absorbing substance is present is also possible. As the amount of absorbing substance decreases, less light is absorbed and more light passes through (see Figure 9-3). When more of the white light passes through the solution, it appears lighter. When wa-

The color of a substance or object is a result of its molecular structure, which results in its absorbing certain wavelengths of light and transmitting others.

Table 9-1

Wavelength (λ) in nm	Color Absorbed	Complementary Color
<380	ultraviolet	none visible
380-435	violet	yellow-green
435-480	blue	yellow
480-490	greenish-blue	orange
490-500	blue-green	red
500-560	green	purple
560-580	yellow-green	violet
580-595	yellow	blue
595-650	orange	green-blue
650-780	red	blue-green

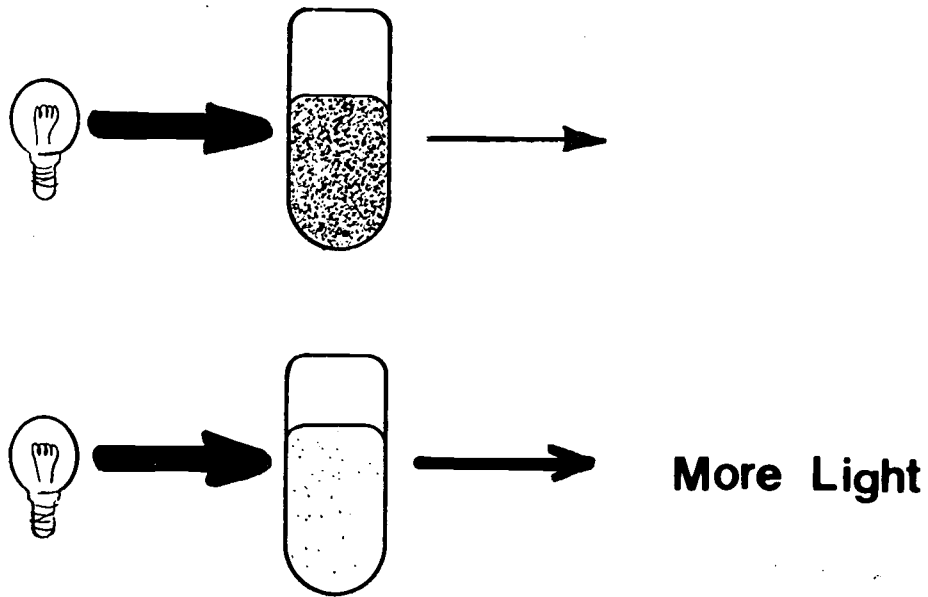


Figure 9-3
Basic absorption.

ter is added to orange juice, the concentration of orange juice decreases, so less greenish-blue light is absorbed and more white light passes through. The color observed then appears pale or weak.

By detecting light's interaction with a substance, identifying and quantifying that substance is possible. These principles elicit interesting questions:

1. If a solution is colorless, such as water, does this mean no light is absorbed?

Yes, except that a substance may be present that absorbs light outside the visible range. Also substances in concentrations too small for the human eye to detect color may be present.

2. If a solution contains many substances that absorb a wide variety of wavelengths of visible light, will the liquid be multicolored?

No. When most or all of the white light is absorbed by the different substances, the solution will appear black.

3. If a solution that appears blue is placed in a room illuminated by only yellow light, its complementary color, what color will the solution appear?

Black. Assuming that the absorbing substance is of a high enough concentration, all the yellow light will be absorbed, and there will be no light transmitted.

Spectroscopic Instrumentation

The principles presented in the previous sections of this chapter have been used to develop instruments that can perform qualitative and quantitative measurements. These instruments are commonly used in such fields as medical research and chemistry. Examples of these types of instruments include spectrophotometers, fluorometers, nephelometers, and turbidimeters. Each uses slightly different principles and differs in design from the rest, but all are capable of identifying and quantitating unknown substances.

Spectrophotometry

Spectrophotometry involves the measurement of mono-

Spectrophotometers quantify the relationships between the colors of an unknown solution or substance and a standard known solution or substance.

chromatic light in the ultraviolet, visible or infrared portion of the electromagnetic radiation spectrum absorbed by a substance in the ultraviolet, visible, or infrared portion of the electromagnetic radiation spectrum. While spectrophotometry is based on the same absorption/transmission theory previously discussed, it is more accurately described and defined by Beer's Law and Lambert's Law. Beer's Law states that the amount of light absorbed is proportional to the concentration of the absorbing substance. Lambert's Law states that the amount of light transmitted is inversely and exponentially proportional to the path distance through the substance. An instrument that employs these principles is referred to as a *spectrophotometer*.

Spectrophotometers have five basic components: light source, monochromator, sample (cuvet), light detector, and read-out device. Other optical enhancements can be added to improve the final readout (see Figure 9-4). The light source is a lamp used to provide white light that can be directed at the next component. The monochromator is an optical device that separates the white light into discrete wavelengths. The monochromator can be moved or rotated to direct a specific wavelength toward the sample that, in turn, can absorb or transmit the light directed toward it by the monochromator. The light detector is an electronic device which measures the amount of light leaving or transmitted through the sample. The detector does not detect the color of resulting light, it only measures the *amount* of light (of any wavelength). The read-out device converts electronic signals from the detector into a readable form, displaying results on an LED panel or scale (on the instrument). Alternatively, a printout may be produced that describes how much light reached the detector.

The orange juice example can be expanded further to explain spectrophotometry. To test the concentration of orange juice, a spectrophotometer could be used. A tube of juice is placed in the spectrophotometer and the monochromator adjusted to allow only the absorbing light to pass through the juice. In this case, remember that blue-green light is absorbed from white light, producing the color orange. If the monochromator is adjusted so that only light of 480-490nm (greenish-blue) strikes the tube of juice and virtually all the greenish-blue light reaches the detector, this would indicate that there is very little absorbing substance (orange juice) in the tube. As the concentration of orange juice in the tube increases, less greenish-blue light reaches the detector (see Figure 9-5). This same type of analysis

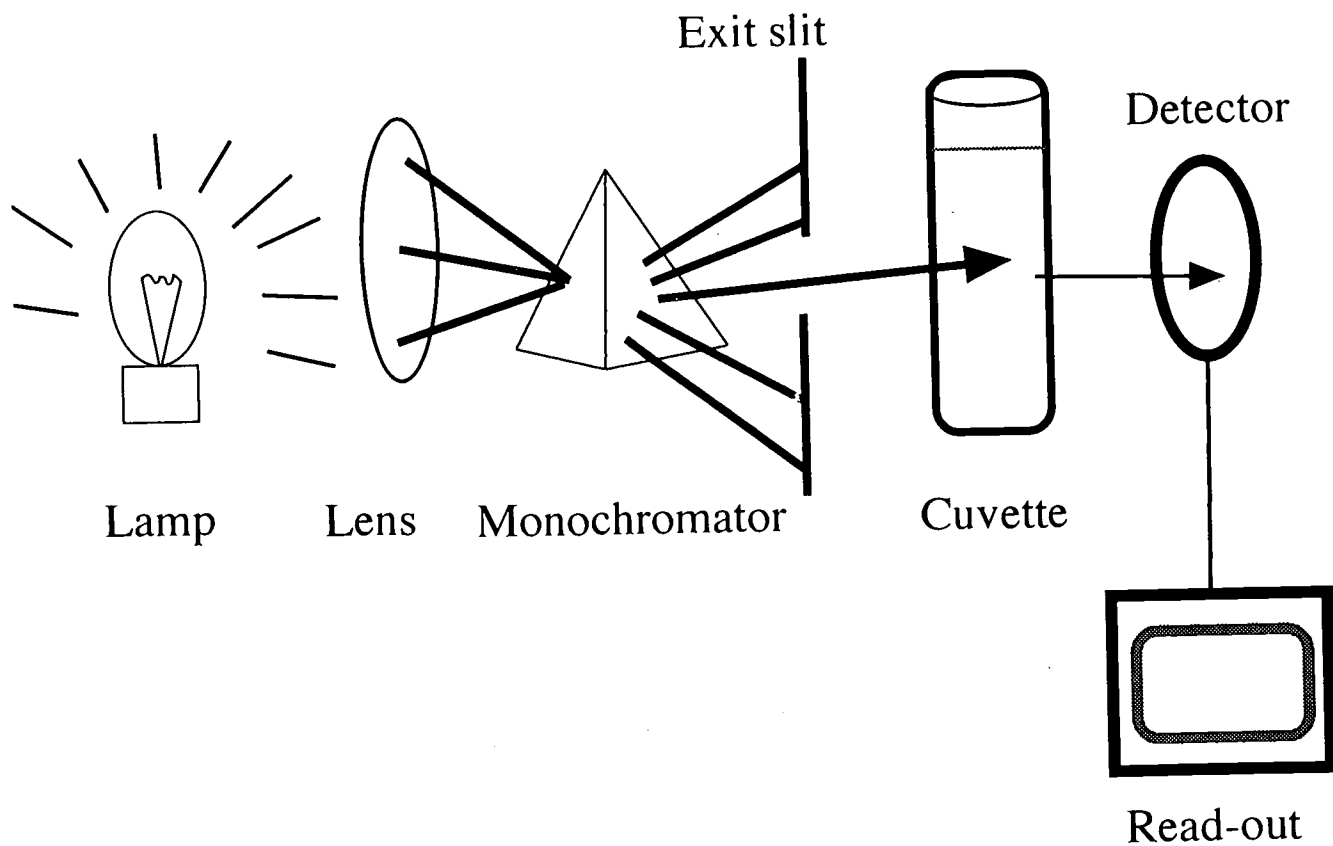


Figure 9-4
Components of a spectrophotometer.

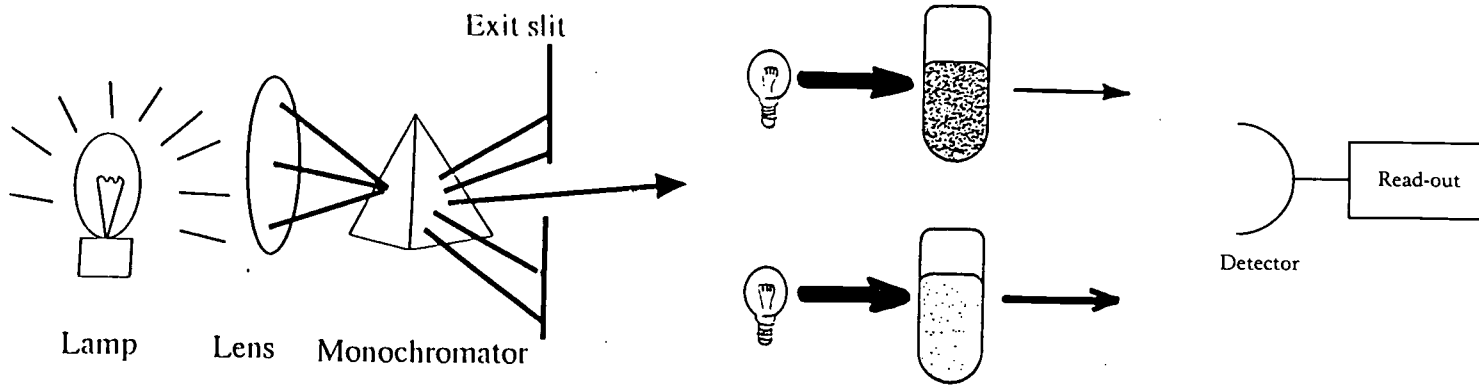


Figure 9-5
Use of a spectrophotometer for analysis of a liquid.

can be used to identify and measure the concentration of virtually any substance with color, whether that color is in the visible light range or not.

Other Instrumentation

Other instrumentation methodologies used today are based on many of the same principles. Examples of such instruments include fluorometers, turbidimeters, and nephelometers. The components of all of these are very similar to those found in the spectrophotometer. The goal of each is quantitation and qualification of various substances.

Fluorometers measure the amount of fluorescence emitted from a substance being analyzed.

Fluorometry is based on the principle that when exposed to light, some compounds absorb that light and then emit light of a shorter wavelength. An example of fluorescence is a toy that, after being left in the sun, glows (or fluoresces) in the dark. The same basic components found in a spectrophotometer can be used to measure fluorescence. The detector, however, must move around 90° because a fluorometer measures the light generated by the compound, not that which comes from the light source.

The more turbid the sample solution is, the less light reaches the turbidimeter's detector.

Turbidimetry is based on the fact that the *particles* in a solution block light transmitted through that solution. The more particles that are in solution, the more light is blocked. The particles block light independent of light wavelength. A turbidimeter is identical to that of a spectrophotometer with the exception that, in a turbidimeter, the wavelength of the light source is not important. This device measures the particles in solution by their ability to absorb and scatter light.

Nephelometry is similar to turbidimetry. It measures reflected light as the light hits particles in solution. Nephelometry is different in that the detector is repositioned 90 degrees, as in fluorometry. The detector must be moved because the nephelometer measures the light reflecting off the particles, not passing through the solution.

Fluorometry, turbidimetry, and nephelometry are all used to quantitate and qualitate substances. A common method used by all three instruments is a process known as *tagging*. In tagging, the substance in question is attached to or tagged with a particle known to either fluoresce or precipitate out of a given solution. Following a series of known reactions, particles will fluoresce, block light, or reflect light. Given these known reac-

tions, the specific instrumentation can be used to measure or identify the tagged substance.

Questions Commonly Asked by Students

Question 1: How can a spectrophotometer be used to identify a substance?

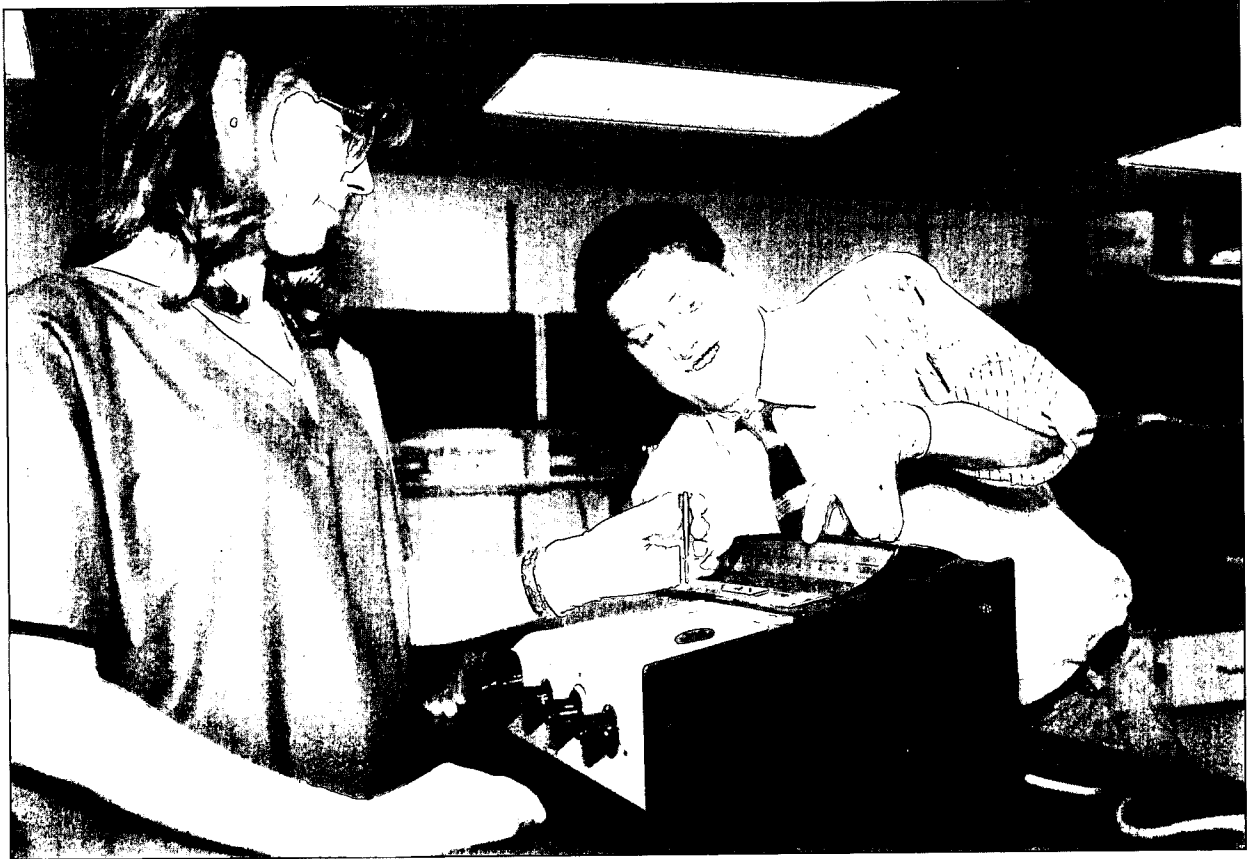
Different substances have distinct absorption curves. These curves identify wavelengths that substance absorbs and those the substance does not. This specific absorption pattern is related to the substance's molecular structure, making identification possible.

Question 2: To what extent are these methodologies used today?

Spectrophotometers are parts of the many instruments used in modern research laboratories. Even though instruments have become sophisticated, spectrophotometers are still important tools. The sophistication relates to speed and efficiency, but the basic instrumentation is the same. A new dishwasher automatically shuts off when the dishes are clean. The dishwasher uses a turbidimetric method to detect when no more food is in the water, indicating that the dishes are clean and the next step in the dishwashing cycle can proceed.

Question 3: If the color or turbidity of solutions appear to be the same, can a spectroscopic instrument tell them apart?

Yes, the detector in the instrument can detect differences the human eye cannot. Color changes occur outside the visible light range that can be detected by spectroscopy.



John Davis discusses with Angela Hernandez, a senior medical technology student at LSU Medical Center, School of Allied Health Professions, the principle of absorption spectrophotometry using a Coleman Junior Spectrophotometer.

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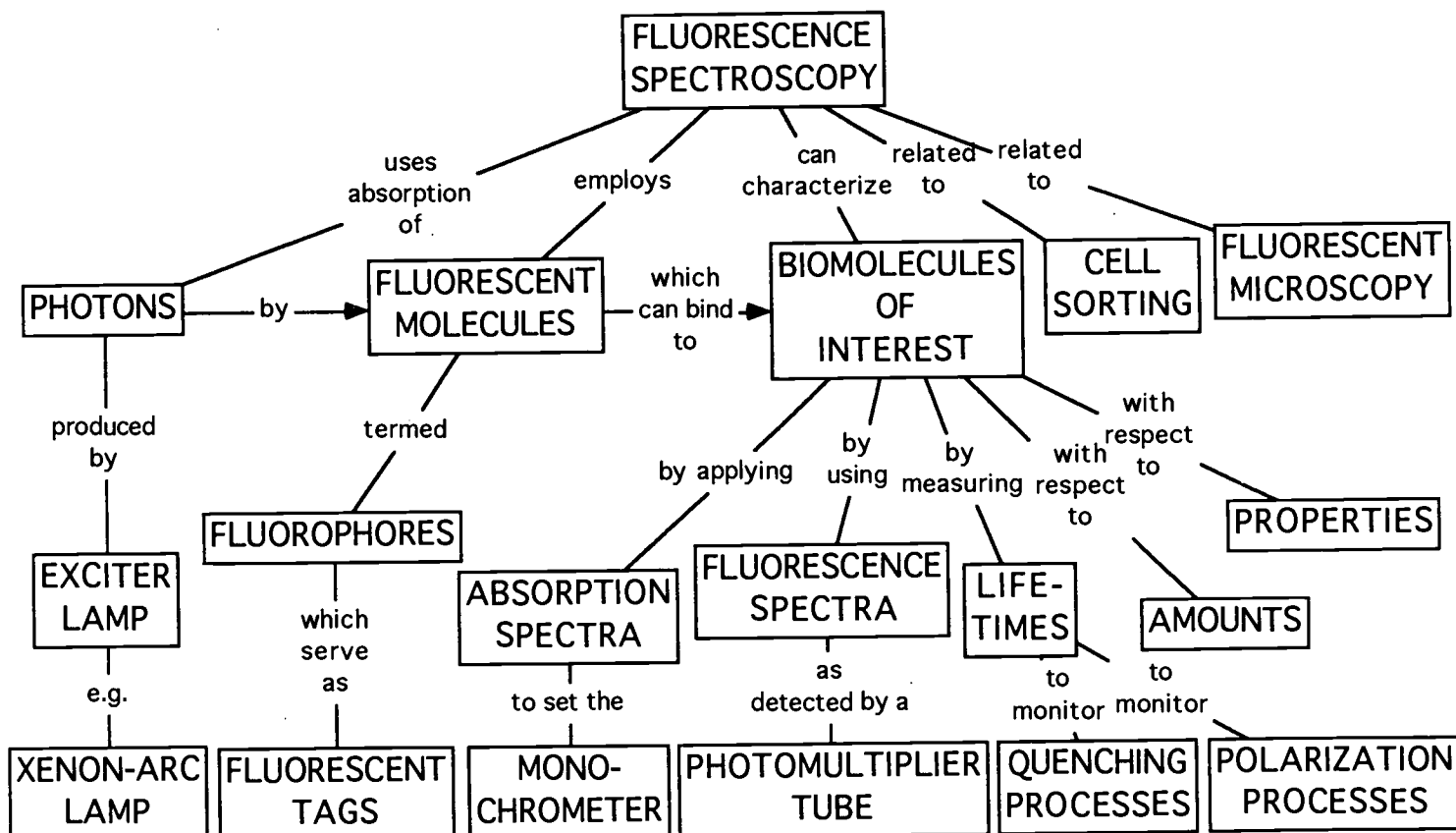
FLUORESCENCE SPECTROSCOPY

10

Alan Abbott

Overview

1. Basic fluorescence measurement
2. Basic instrumentation
3. Fluorescence techniques
4. Fluorescence anisotropy
5. Fluorescence quenching and energy transfer
6. Specialized instrumentation
7. Lifetime measurements



Fluorescence Spectroscopy

Introduction

Fluorescence spectroscopy has the advantages of sensitivity and specificity, allowing it to be used for detection and characterization of molecules in trace amounts.

A fluorophore is a molecule that emits light in response to light absorption.

*Nano = billionth
Pico = trillionth*

Three common techniques used to determine the physical properties of the fluorescent molecule are:

- 1) polarization*
- 2) collisional quenching*
- 3) quenching due to energy transfer.*

Fluorescence spectroscopy is done with an instrument called a fluorescence spectrometer.

Fluorescent light is the result of the absorbance of a photon of radiant energy by a molecule and re-emission of a second electron.

The energy of the light associated with fluorescence is characterized by its wavelength.

Fluorescence spectroscopy is useful for quantitating and characterizing molecules in trace amounts. Generally, a fluorescent molecule, which is commonly referred to as a fluorophore, is one that emits light in response to light absorption. The blue glow of gasoline exposed to sunlight and the vivid colors observed under a black light from dyes in clothes are everyday examples of fluorescence. In a fluorescence spectrometer, the emitted light (or fluorescence light) is detected at a geometry and wavelength that eliminates background excitation light. This enhances the detection sensitivity to the point that molecules can often be detected in nanomole to picomole amounts.

Several aspects of the fluorescence process can also be manipulated to obtain the physical properties of the fluorescent molecule. One is *fluorescence polarization*, a technique that ascertains the relative rotational motion of the fluorescent molecule. A second is *fluorescence collisional quenching* that is used to ascertain solvent accessibility of a fluorophore. A third is *fluorescence quenching due to energy transfer*, where the distance between two fluorophores is measured. These are some of the more commonly used techniques.

This review will describe the basic phenomenon of fluorescence and some of its applications. Basic instrumentation will be discussed, as well as brief descriptions of more sophisticated extensions. These topics will be treated on a very descriptive level, but details required for actual fluorescence studies are readily available (see suggested readings at the end of this chapter).

Basic Fluorescence Measurement

What is *fluorescence*? Fluorescence occurs when a photon of light excites an electron to a higher energy state and a second photon is re-emitted upon the electron's return to the original "ground" state. This energy difference normally can only be a certain value (it is "quantitized") that must be matched by the photon energy. The photon energy (E_p) required is described by its wavelength (λ):

$$E_p = hc/\lambda$$

where h and c are constants. This equation shows that wavelength is inversely proportional to photon energy.

The light absorbed is referred to as absorbance, and the light emitted is referred to as fluorescence.

The preceding description implies that fluorophores absorb and emit light of the same energy. Figure 10-1 shows this is not the case. The intensity of light absorbed (absorbance) and emitted (fluorescence) is plotted versus wavelength for a common fluorophore, *fluorescein*. Note that light absorption occurs at higher energy (shorter wavelength) than light emission. Also, the broadness of both plots indicates that transitions of more than one wavelength occur. These features are common to all fluorophores in liquid solvents.

As a result of light absorption, a molecule is excited and is raised from ground state to a higher energy level.

The explanation for the above behavior lies in the stretching and bending of the chemical bonds within the molecule. These motions create a series of vibrational states with varying energy levels (see Figure 10-2). The broadness of the absorption spectrum is explained by allowing part of the photon energy to be used to attain the higher energy vibrational states. Now photons can be absorbed that have the energy of a vibrational state transition. Generally, electrons excited to higher vibrational levels quickly return to the lowest vibrational state prior to fluorescence. Necessarily, the transition to ground state will be accompanied by the emission of light energy less than or equal to that of the excitation light. The emission spectrum's broadness is also explained by considering vibrational energy states. The transition of excited electrons to multiple vibrational levels of the ground state molecule (see Figure 10-2) causes the release of a mixture of photons whose energies are separated only by ground vibrational energies.

As excited electrons drop from a higher energy level to a lower energy level, energy is released in the form of new light (fluorescence).

This model suggests several practical considerations in measuring fluorescence. For example, the sample can be excited at one wavelength and fluorescence recorded at another. This is especially important since most aqueous or solid samples will scatter light in greater quantities than the light of fluorescence. But that scattered light will almost exclusively be at the excitation wavelength, whereas fluorescence light can be measured at a completely different wavelength. Another result of the model is that the fluorescence spectrum is not strongly dependent upon a specific absorption wavelength. One can generally excite the fluorophore at any wavelength within the absorption spectrum and the resulting fluorescence "emission" spectrum will always look the same. It is, therefore, diagnostic for a particular fluorophore and can be measured using the most convenient excitation wavelength. Finally, relative changes in fluorescence intensity will be the same throughout the emission spectrum. Thus, there is no need to measure the whole spectra to monitor relative changes in fluorescence intensity.

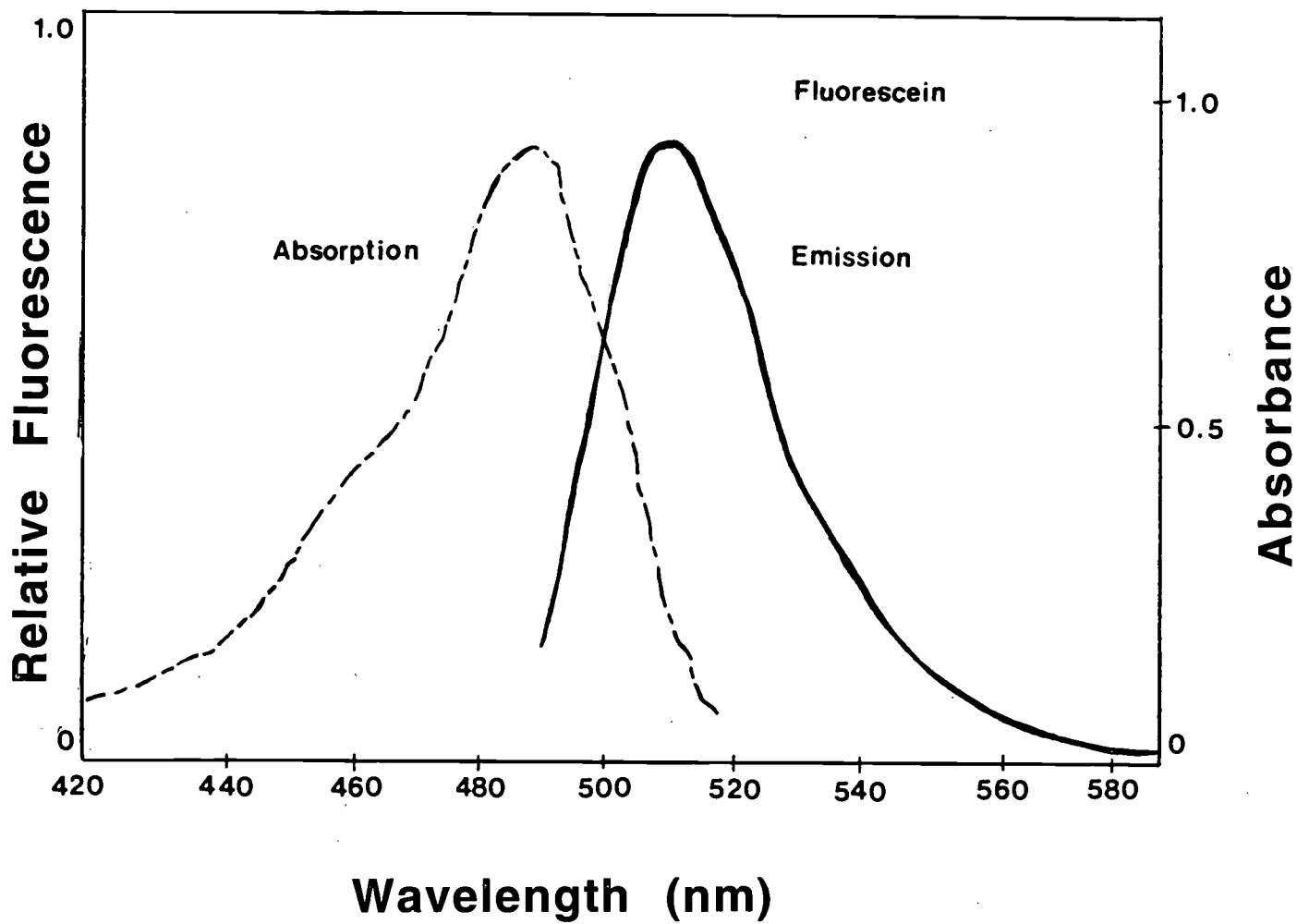


Figure 10-1
Absorbance and fluorescence.

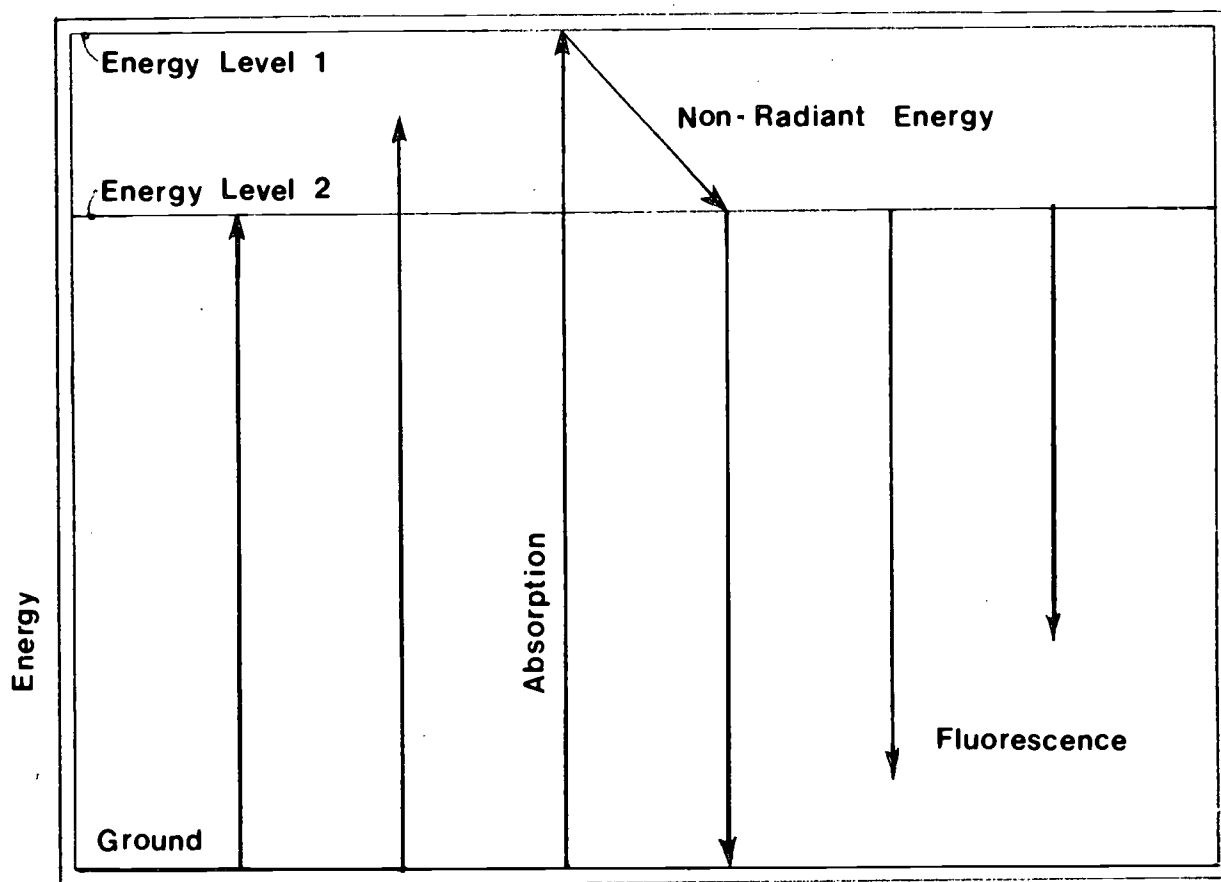


Figure 10-2

Both absorption and fluorescence spectra are broadened by the multiple transition energies available from closely spaced vibrational energy states. Both absorption and fluorescence transitions occur from their lowest energy vibrational state. The fluorescence transition is the slowest and therefore rate limiting.

A fluorophore can be excited at any wavelength in the absorption spectrum but the resulting fluorescence spectrum will always look the same.

Influences on the intensity of fluorescence include the following: changes in pH may induce changes in the ionic state of the molecule, thus changing fluorescence. Increasing the temperature of the substance increases molecular motion, thus increasing molecular collisions and decreasing fluorescence. An increase in concentration of the substance or solution decreases the degree of fluorescence due to the effect of more concentrated molecules on absorption of emitted light. These factors combine to alter the theoretical linearity of fluorescence

Instrumentation

The *basic fluorescence spectroscopy instrument* is shown in Figure 10-3. It consists of a light source focused on a sample chamber with either a filter or monochromator in between to select a particular excitation light wavelength. The sample chamber is temperature controlled and isolated from room light, and it usually accepts a cuvette having a one to three milliliter capacity. Unlike the excitation beam, the emitted light shines equally in all directions. It is, therefore, measured at a 90° angle from the excitation light to eliminate background excitation light. The emitted light also passes through a filter or monochromator used to select the emission wavelength, and it is detected using a high voltage photomultiplier tube. Relative fluorescence is simply recorded in terms of electric current from the photomultiplier tube. Current fluorimeters of this type typically have these components largely under computer control and can be purchased for \$12,000 to \$25,000 at this writing.

Fluorescence is recorded as an electric current from the photomultiplier tube

In order to cover a wide range of fluorophores, the optimal light source should cover the 200 to 800 nm wavelength range. None does entirely, but one of the best sources is the *xenon-arc lamp* (300-700 nm). Of lower intensity but similar wavelength range is the halogen lamp. Less widely used is the *high-pressure mercury lamp*.

Two monochromators or filters are used with fluorescence spectroscopy:

- 1) one to regulate the wavelength of the light striking the sample, and,*
- 2) one to isolate the wavelength emitted from the sample.*

In order to select the desired excitation or emission light, glass filters that allow mainly the desired wavelength to pass through may be used. These, however, need to be switched for different fluorophores. A much more convenient method is to use a *monochromator*. Light entering this device is separated using a diffraction grating. Gratings are pivoted (often by a motor that is under computer control) in order to cause the desired wavelength to shine out of the monochromator and into the

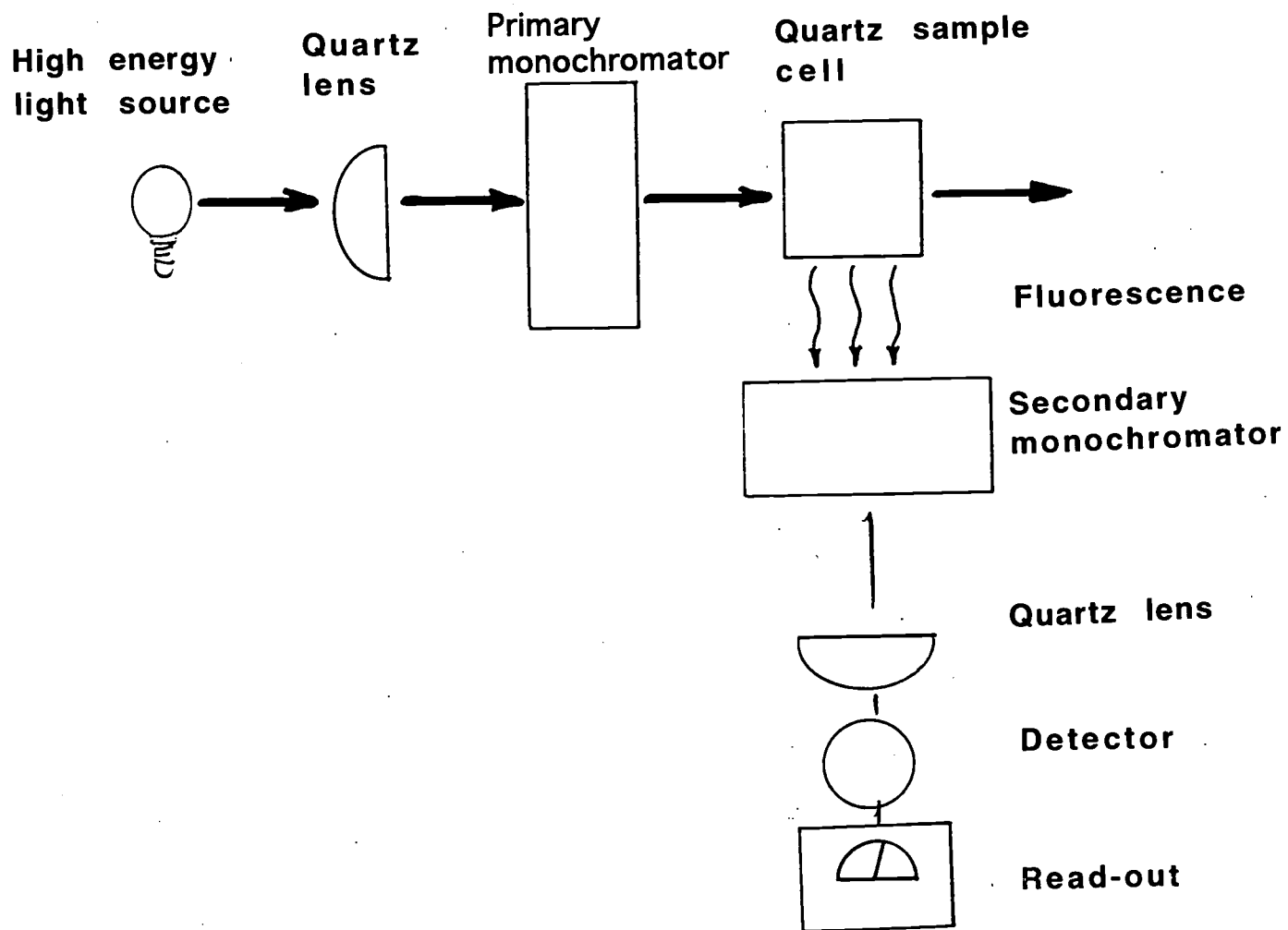


Figure 10-3
Basic fluorescence instrument.

sample compartment. Generally, the exit slit can be adjusted to allow a band of wavelengths (0.3 -16 nm wide) to shine out.

Because low levels of light or quick bursts of light must be measured, a photomultiplier tube is used to increase the detection sensitivity.

Photomultiplier tubes detect light by converting it to electric current. They are sensitive devices that can be damaged by excessive light. An electrical voltage is applied across two metal plates and current is generated by a photon hitting the negative plate and ejecting an electron toward the positive plate. The voltage applied across the plates is adjustable with the higher voltages being more sensitive to light.

In order to measure relative amounts of a fluorescent molecule, the operator sets the excitation and emission wavelengths as close to the absorption and emission maxima as is practical. Some precautions are necessary to observe. To keep fluorescence response linear with fluorophore concentration, set the sample absorbance at 0.05 or less. This prevents the lowering of excitation light intensity as the light passes toward the back of the sample (inner filter effect). To prevent the loss of fluorescence due to light intensity depleting ground state fluorophores or chemically degrading them (photo bleaching), excitation slit widths are not opened wider than is necessary.

Maintain excitation slit width as small as necessary to prevent loss of fluorescence.

Fluorescence Techniques

Most biological molecules are not fluorescent. This allows the detection of fluorophores in a heterogeneous biological sample. Great effort has, therefore, gone into the development of fluorescent molecules that bind specifically to the biomolecule under study. One way to do this is to chemically link a fluorophore to another molecule that has the desired binding affinity. Other fluorophores simply bind noncovalently to particular molecules.

Fluorescent immunoassay is an example of chemically linking a fluorophore. It requires purified, specific antibodies, which are biomolecules produced in higher animals in response to infections that specifically bind to "non-self" molecules. Antibodies that bind exclusively to a molecule under study can be raised in laboratory animals through this process and purified either from the animal's blood or from its antibody-producing spleen cells. These antibodies are then fluorescently tagged so that when they bind to their target they can be detected. A typical tag is *fluorescein isothiocyanate*. The fluores-

Fluorescence immunoassay has about one-tenth the sensitivity of radioimmunoassay, but does not require radioactive material.

cein group is a well-known fluorophore, and the isothiocyanate group is reactive toward nucleophilic amino acids (e.g., lysine) found in the antibody. There are other tags that combine other fluorophores with reactive groups. The covalently labeled antibody is added to the solution to be assayed and allowed to bind to any antigens present. Conditions are usually such that the antibody will be in excess over the antigen, and the excess unbound antibody is removed prior to quantitation.

Cancer cells, for example, often have an identifying antigen on their surface. Antibodies specific for this antigen and tagged by fluorescein can be added to a suspension of these cells and then the excess is removed by collecting cells on a filter that allows antibodies to pass through. The amount of antibody bound to the cell, and thus the amount of cancer-associated antigen, is detected by fluorescence. There are numerous examples of fluorescent immunoassays used to detect everything from genes in cellular DNA to blood toxins to environmental pollutants (see Diamandis, 1993).

Many noncovalently bound fluorophores are also used because of their particular affinity for other molecules. Distinction will be drawn here between polarity sensitive fluorophores, ion selective fluorophores, and affinity analogs. Polarity sensitive fluorophores are generally soluble in aqueous solutions but partition much more strongly into hydrophobic solvents. In addition, their fluorescence is usually enhanced several fold in the hydrophobic phase.

Deoxyribonucleic acid, for example, may be quantified by fluorophores that are hydrophobic, planar, and positively charged, such as ethidium bromide. These dyes literally insert or "intercalate" between deoxyribonucleotides in the DNA sequence. Lipid membranes, on the other hand, strongly bind diphenylhexatriene (DPH) and its derivatives. These fatty acid-like molecules easily partition into phospholipid membranes. Changes in physical properties specifically of the membrane, like a liquid-to-solid phase transition, can often be detected by a change in the fluorophore's fluorescence. Proteins also have hydrophobic molecular interiors and the fluorophore 1,8-ANS detects proteins selectively. Changes in protein structure often affect the fluorescence of this molecule, and this can be used as an indicator of the structure change.

Ion selective fluorophores bind cations or anions and are selective enough to be ion specific sensors. Fluorescence intensity is enhanced by the binding process. In these studies, the fluorophore is limiting, and its fluorescence increase is correlated with the ion's concentration. Intracellular calcium levels have been monitored using FURA-2. This fluorophore may be introduced into living cells and used to monitor calcium metabolism directly.

Affinity analog fluorophores are those that behave chemically like the molecule under study but are also fluorescent. These are numerous, but TNP-ATP will serve as a representative example. Many enzymes of interest to biochemists hydrolyze the high-energy compound ATP and can be isolated to homogeneity. The ATP molecule covalently linked to the fluorophore trinitrophenol (TNP) selectively binds to these proteins and is fluorescent. The fluorescence is enhanced by the binding, so the degree of enhancement can be used to quantitate the number of enzymes present. A related type of affinity analog is the fluorescent enzyme substrate. Because enzymes catalyze very specific chemical reactions, their substrate may be attached to a fluorophore. Commonly, the fluorescence of the analog changes when it is converted to product. Here enzyme amount may be quantitated by the rate of fluorescence change.

Affinity analog fluorophores have fluorescence and behave chemically like a molecule of interest.

Fluorescence Anisotropy (or Polarization)

In addition to the detection of fluorescence intensity, a fluorophore's relative rotational motion may be measured. This is a result of two principles of the fluorescence process: (a) light travels as waves that only excite fluorophores aligned correctly with the wave, and (b) a delay of 1-10 nanoseconds usually separates the light absorption and emission processes. This allows time for the fluorophore to reorient in space. To measure the degree of reorientation, the excitation light is passed through a polarizing filter that only allows vertically polarized light to hit the sample (see Figure 10-4). If the fluorophore is immobilized, then the emitted light will also be vertically polarized. A decrease in vertically polarized light emission, accompanied by an increase in horizontally polarized emission, indicates some reorientation prior to emission. This effect may be quantitated by calculating the *anisotropy* of the fluorophore:

$$\text{Anisotropy} = (I_v - I_h) / (I_v + 2I_h)$$

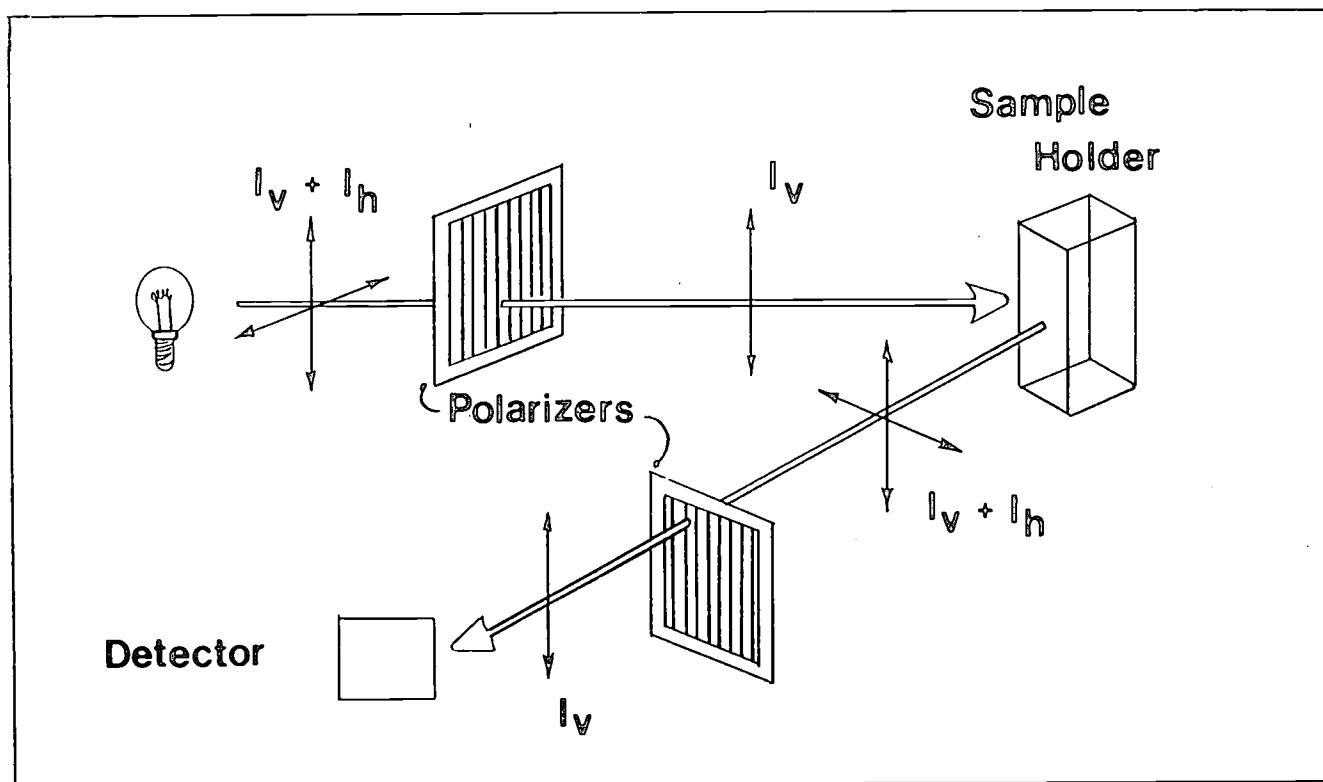


Figure 10-4

Anisotropy measurement. The degree to which vertically polarized excitation light is still vertical upon emission is measured by comparing I_v and I_h on the emission side.

When fluorescence molecules are excited with polarized light, they emit partially polarized light with an intensity directly related to the degree of rotation of the molecule.

Anisotropy is used in immunoassay for the detection of a drug or natural metabolite from human or animal fluids.

where I stands for intensity, v for vertical, and h for horizontal. For clarity, the instrumental correction factor has been left out of this equation. Molecules of less than 1000 MW often completely randomize their orientation prior to fluorescence and $I_v = I_h$, thus the anisotropy is zero. Whole cells, on the other hand, have very little rotational motion during 1-10 nano-seconds and may completely immobilize a fluorophore. This anisotropy is usually near 0.40. Fluorescence anisotropy has been most useful in measuring the binding of small molecules to their biological binding sites on relatively large molecules.

Anisotropy has been very useful in immunoassay for the detection of drugs or natural metabolites in biological fluids. In this case, a fluorescent metabolite is made by reaction with a fluorophore. Its anisotropy is usually 0.0. It is added to enough antibody to bind up most of it and increase the anisotropy to the 0.1-0.2 range. The test solution is then added. A positive test for the antigen is a decrease in anisotropy due to competitive displacement of some fluorescent antigen from the antibody. Because this immunoassay requires no separation steps (i.e., separation of unbound antibody), it is both simple and sensitive.

Solution viscosity affects the fluorophore reorientation rate and, therefore, influences anisotropy. The viscosity of biological membranes has probably received the most attention. Temperature, the degree of membrane fatty acid unsaturation, and cholesterol content (mainly) affect the viscosity of membranes. The anisotropy of DPH and its derivatives increases with viscosity, due to less rotational motion between light absorption and emission. Using these probes, cellular reactions in membranes have been found to be highly dependent on viscosity. Furthermore, cells carefully regulate cholesterol and fatty acid content in order to maintain a particular membrane fluidity. These probes can be used to show that organic solvents, such as ethanol, increase membrane fluidity and probably contribute to its intoxicating effects.

Fluorescence Quenching and Energy Transfer

Quenching is the process of deactivating molecules from the excited state without the emission of light via the presence of a chemical or colored compound.

A fluorophore on a biomolecule of interest, upon photon absorption, can lose this excitation energy through a variety of non-radiant processes. Then its fluorescence intensity decreases, and it is said to be *quenched*. Different types of quenching are often induced because they give information on the fluorophore's physical or chemical environment. *Collisional*

Collisional quenching occurs when high molecular weight atomic ions or certain uncharged molecules collide with the fluorophore.

quenching, for example, occurs when high molecular weight atomic ions, typically I or Cs⁺, or certain uncharged molecular compounds (e.g., oxygen or acrylamide) collide with the fluorophore. They collide by diffusional encounters with the fluorophore during its excited lifetime (1-10 ns). Fluorophores that are buried in the matrix of a biomolecule can then generally be distinguished from solvent accessible fluorophores by their lack of significant quenching.

Another type of quenching is referred to as *fluorescence resonance energy transfer* or FRET. Under specific conditions this technique measures the distance between two fluorophores separated by 10-100 angstroms. One requirement is that the energy of photon emission from a "donor" fluorophore exactly equals the energy of photon absorption by an "acceptor" fluorophore. This happens when donor emission spectrum and acceptor absorption spectrum overlap. When the fluorophores are in close proximity and properly oriented, donor fluorescence is quenched by the acceptor. This causes excitation of the acceptor, which then results in acceptor fluorescence. This effect is shown in Figure 10-5 for the fluorescein, Erythrosin donor-acceptor pair. The top plot confirms that Erythrosin absorbance overlaps the fluorescein fluorescence spectrum. The bottom plot shows the donor emission spectra before and after addition of the acceptor. Although the donor fluorescence is clearly quenched, a hump on the quenched spectrum arises from the additional acceptor fluorescence now present. These data were used to measure the distance between ATP binding sites in an enzyme dimer. It is one of several FRET distances that have placed limits on models of the enzyme's structure.

Another common use of FRET is the binding of donor- and acceptor-labeled molecules. This is often used to monitor proteins binding to biological membranes. Proteins have an intrinsic fluorescence by virtue of their tyrosine and tryptophan amino acids. They behave as donors while fluorophores incorporated into the membrane behave as acceptors. In this way, protein-membrane assemblies of membrane associated processes (e.g., blood coagulation, complement activation) have been studied.

Specialized Instrumentation

This chapter has emphasized basic fluorescence theory,

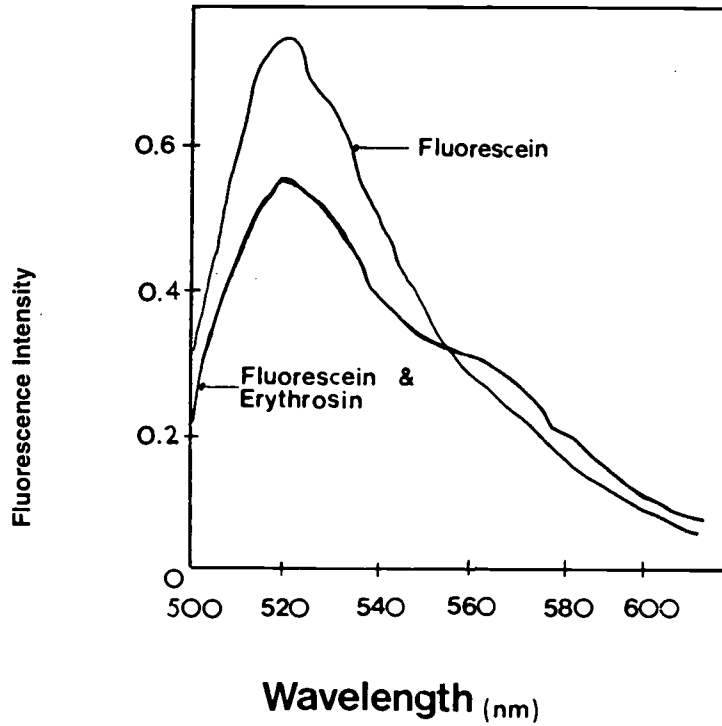
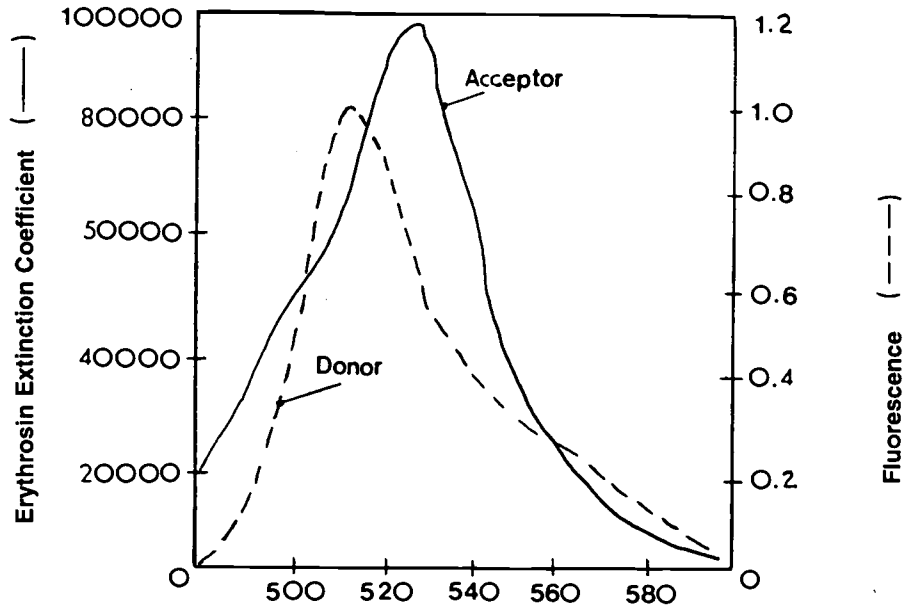


Figure 10-5
Excitation of the acceptor resulting in fluorescence.

Fluorescence microscopy and fluorescence cell sorting are two methods to study cell structure and metabolism.

instrumentation, and techniques. It is important to mention briefly some of the more sophisticated extensions of these techniques because they are the basis of exciting research in biology. *Fluorescence microscopy* and *fluorescence cell sorting* are techniques extremely important to the understanding of cell structure and metabolism. With a fluorescence microscope (see Chapter 6), like the basic fluorometer, the excitation light is trained on a sample (which is now a microscope slide with this instrument), and fluorescence is actually detected 180° from the direction of the excitation light. Monochromators do not fit in the microscope, so filters select excitation and emission wavelengths. Finally, eyesight observations or photographs of the fluorescent cells substitute for photomultiplier detection.

Many protein macrostructures, such as the cell cytoskeleton, have been "lit up" by decorating them with multiple fluorescent antibodies. Also, cell surface antigens have been localized to cell-cell contact regions and other specific locations, often for unknown metabolic functions. More recently, methods for fluorescently labeling individual genes within the cell genome have allowed researchers to count the number of copies of a specific gene that exist in individual cells.

Fluorescence cell sorting is a technique that passes cells in solution through an excitation light beam and measures fluorescence emission intensity. Passing large populations of cells individually through the light allows the accumulation of fluorescence data from the entire cell population. In addition, in-solution conditions allow taking routine measurements from whole populations of living cells.

Lifetime Measurements

"Lifetime" is referred to as the delay between absorbance and fluorescence.

The delay between absorbance and fluorescence is referred to as its *lifetime* or *mean life*. The instruments described so far continuously excite the fluorophores and, therefore, have a continuous fluorescence emission. Nonetheless, the quenching and polarization processes described previously can all be monitored by measuring lifetimes. The lifetime can be measured by flashing the sample with excitation light and then measuring the time required for fluorescence to appear. Both quenching mechanisms already mentioned simply lower the lifetime in proportion to the decrease seen for continuous fluorescence. Anisotropy can also be measured.

The reason to make lifetime measurements is to more carefully delineate fluorescence from extraneous light signals. In biological samples, for example, two or three lifetimes are sometimes found. In addition, a very short lifetime (< 0.5 ns) is often contributed by the scattering excitation light. The light scattering is artifactual, but the other lifetimes indicate the presence of more than one fluorophore labeling site. The lifetime measurement allows one to see how fluorescence changes (e.g., quenching) affect each lifetime component. A much more realistic, although often more complex result is obtained by measuring lifetimes.

In summary, fluorescence spectroscopy may be used to quantify trace amounts of fluorescence compounds or other substances that may have fluorophore tags, such as bound fluorescent antibodies. These experiments often have detection limits of 1 nanomole to 10 picomole; which are surpassed only by radioactivity based methods. The protocol is often very simple, such as addition of ethyidium bromide to a solution of DNA and measuring fluorescence intensity. Alternatively, fluorescence may be used to monitor changes in a macromolecule's structure (often it's a biomolecule) through the fluorescence changes of a fluorophore linked to the macromolecule. Thus, small changes in the molecule can be measured using nanomolar concentrations of it. This has been very useful in monitoring the interactions of small molecules (e.g., drugs) with their biological receptors (often specific proteins). It has also been used to detect changes in protein, DNA, and cell membrane structure in response to physiological changes (e.g., solution calcium concentration). In either analytical or basic research laboratories, fluorescence measurements often offer the quickest and most easily obtained information about molecules.

Questions Commonly Asked by Students

Question 1: How much of the fluorescent molecule is needed?

Most experiments are conducted with 10 nanomolar to 1 micromolar concentrations, using a 1 milliliter sample.

Question 2: Which solution variables are the most important to control during an experiment?

Temperature should be maintained within 1° C. Fluorophore absorbance should be 0.05 absor-

bance units or less to avoid a nonlinear response of fluorescence to concentration. The higher the concentration of dissolved salts and buffers, the more likely fluorescence will be partially quenched.

Question 3: Which instrument variables are the most important to control during an experiment?

The variables of wavelength and emission should be controlled. These should be set within absorption and emission spectra, respectfully, and separated by at least 10 nm. Slit widths can be increased to enhance the intensity of the fluorescence and decreased to reduce it. The photomultiplier voltage should be set appropriately. If set too high, the emitted light will be off scale and cannot be quantified. If set too low, emitted light will be unreadable.

Question 4: When should I use fluorescence?

Fluorescence is used for quantitating nanomole amounts of DNA. It can be used to monitor molecular changes in cell membranes and isolated proteins. It is also used to detect nanomole-picomole amounts of any molecule that is the target of an antibody and to quantitate the amount of an enzyme using fluorescence substrates.



Dr. Abbott explains fluorescence spectroscopy to Mike Morris, a senior cardiopulmonary science student at LSU Medical Center, School of Allied Health Professions, Shreveport, LA.

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About the Author

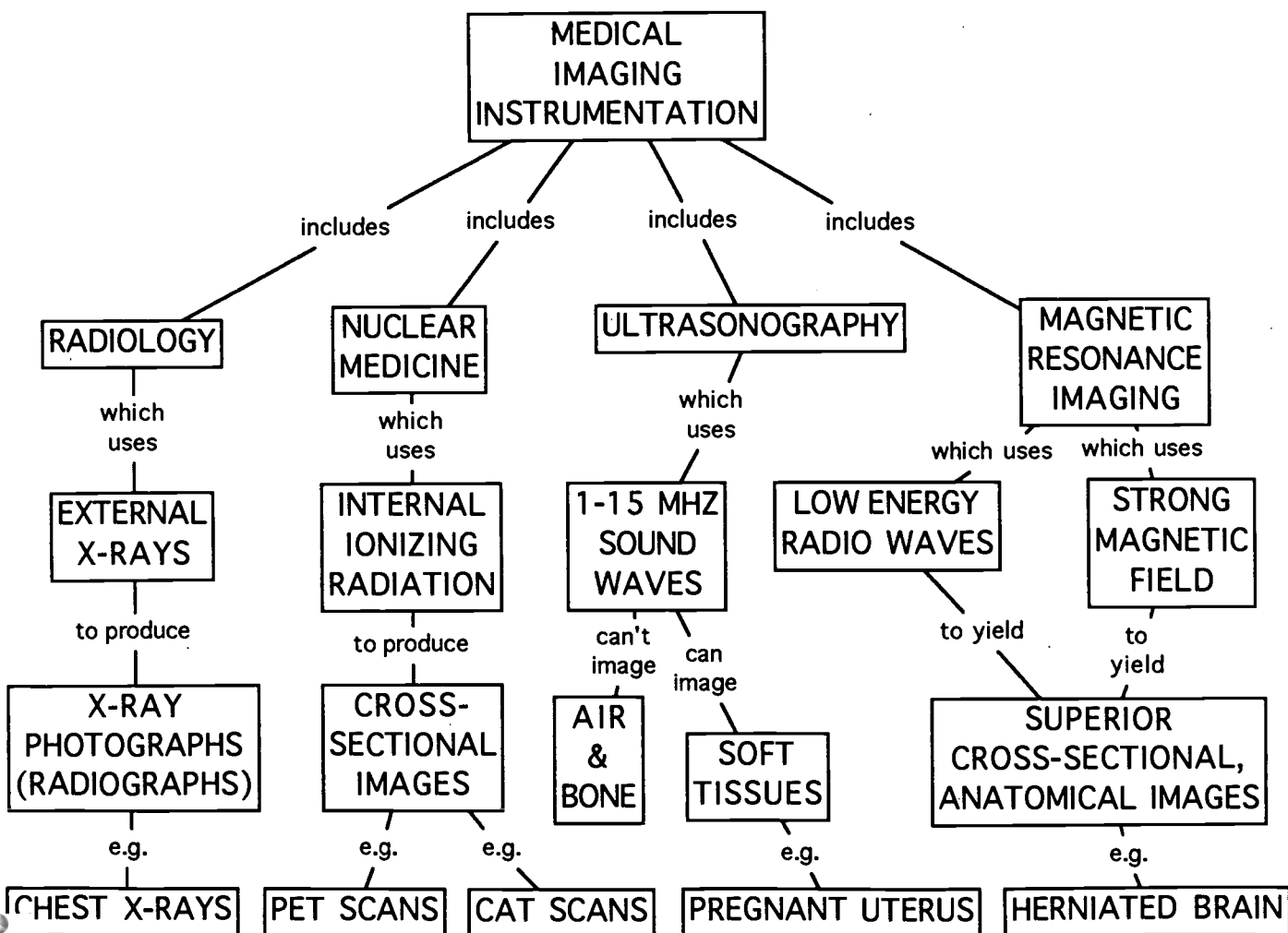
Alan Abbott, Ph.D., is an Assistant Professor of Chemistry at Louisiana State University in Shreveport, LA. His graduate and postdoctoral studies were in biochemistry at the University of Minnesota and University of Cincinnati, respectively. His research emphasis includes protein structure and protein-membrane interactions using fluorescence techniques for investigating blood coagulations and heart disease. He is currently investigating how new members of the cardenolide class of plant toxins affect the enzymes Na^+ , K^+ ATPase.

CROSS-SECTIONAL MEDICAL IMAGING

Mardjohan Hardjasudarma

Overview

1. Computed tomography
2. X-rays: Production and transmission
3. Conventional tomography
4. Analog and digital imaging
5. Single photon emission computed tomography
6. Positron emission tomography
7. Ultrasonography
8. Magnetic resonance imaging
9. Image interpretation



Cross-Sectional Imaging

Since time immemorial, healers of disease have wanted to look into, or through, the human body in order to determine what kinds of ailments had befallen their patient. In the past, this could only be done by visual inspection at surgery or by studying an organ or body part after its removal from the patient. Naturally, the entire body could be studied after death, at an autopsy, when it was obviously no longer beneficial to the deceased. This situation changed dramatically with the discovery of some unusual rays by Wilhelm Conrad Roentgen in Germany in 1885. The mysterious nature of the rays caused them to be called *X-rays*, a term still popular and widely used today. These rays, which for the first time in history enabled visualization “through” the body, are also known by their inventor’s name, *Roentgen rays*.

This led to the development of *radiology*, a branch of medical science that studies and interprets images of the human body created by X-rays on photographic film. Initially, all images were a composite of shadows of structures that the X-rays had traversed. A good example is the human chest X-ray, which clearly shows the shadows of the heart, the lungs, the ribs, and the soft tissues, on top of one another, on a *two-dimensional* piece of film.

Important as transmission imaging remains, attempts were also made at *cross-sectional imaging*, or *tomography*. Specialized machines were developed to use X-rays to create cross-sectional images without the help of computers, a method called *conventional tomography* (see Figure 11-1A and 11-1B). The advent of the computer made possible a more advanced method called *Computerized Axial Tomography* (also known as “CAT scan”). As opposed to radiography, which uses an external source of X-rays, nuclear medicine (NM) involves internal placement of a radioactive substance, usually by injection, ingestion, or inhalation. In nuclear medicine, therefore, the patient becomes the source of ionizing radiation. Specialized NM machines using computers can produce cross-sectional images, a method called *Single Photon Emission Computed Tomography* (SPECT). A variant thereof is known as *Positron Emission Tomography* (PET).

Radiological science is now often called *medical imaging*, since some modalities now in use do not utilize X-rays or ionizing radiation at all. One method uses ultrasound waves

Radiographic imaging uses X-rays to differentially penetrate the body and produce 2-D photographs of various anatomical structures.

Computerized axial tomography (CAT scan) uses a computer to analyze absorption of X-rays by various body structures to reconstruct a cross-sectional image of the body on a television screen. Hundreds of cross-sections can be combined to form a 3-D image of various anatomical structures (e.g., thorax).

Nuclear medicine aids imaging the body via placement of internal radioactive substances.

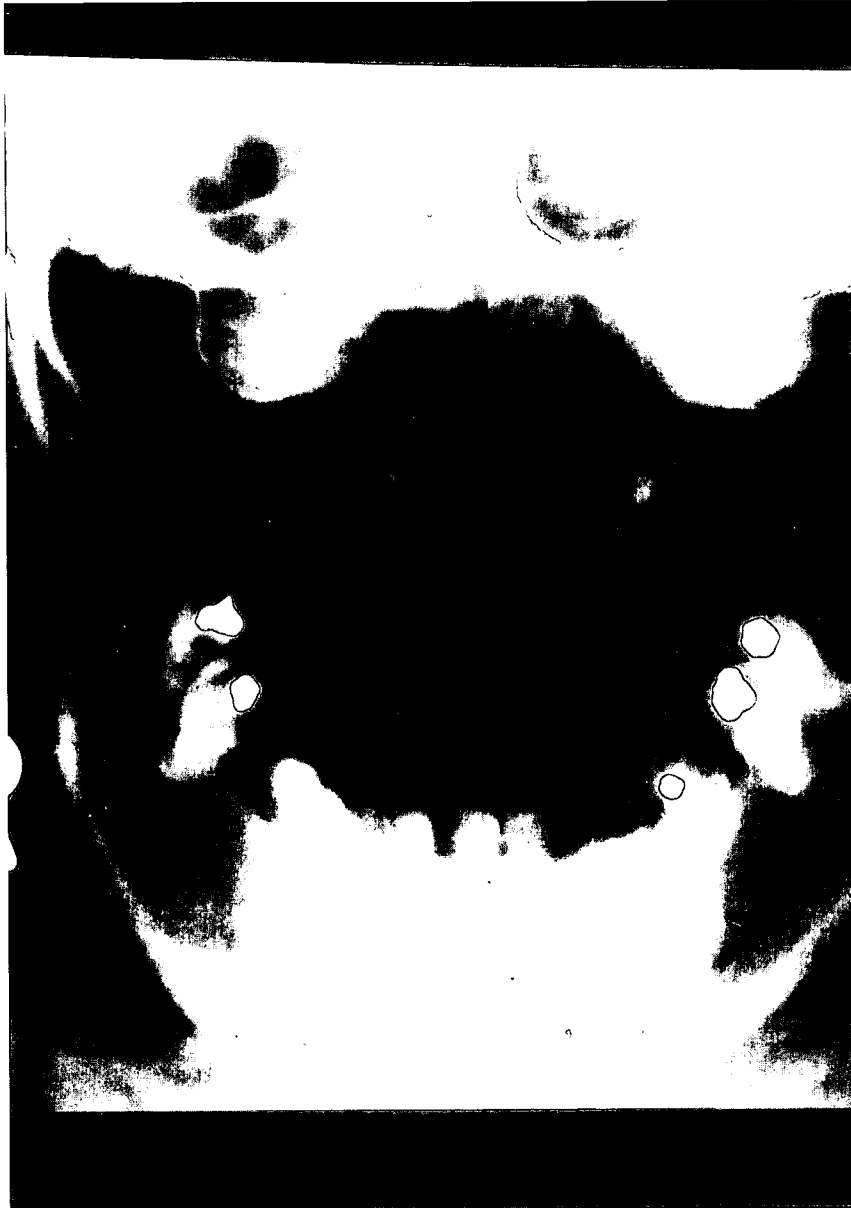


Figure 11-1A

Standard anterior-posterior radiograph (X-ray) of the neck fails to show fracture of the atlas (arrow indicates position of fracture). Figure 11-1B on the following page clearly reveals the fracture.



Figure 11-1B

Conventional tomogram of the neck shows a cross-section of the first two vertebrae — exhibiting a fractured atlas (see arrow).

and is called *ultrasonography* (US). Another is based on use of a magnetic field and radio frequency waves. It is called *Magnetic Resonance Imaging* (MRI). Both US and MRI employ computers to produce and store images.

X-Rays: Production and Transmission

X-rays and gamma rays occupy the upper end of the electromagnetic spectrum. They have the highest frequency, greatest photon energy, and shortest wavelength. X-rays are created by taking energy from electrons and converting it into photons. (A photon is a quantum of light energy.) X-rays are produced in an evacuated tube that contains an anode and a cathode connected to a high-voltage source. When the power supply is switched on, it heats the cathode causing the production of (negatively charged) electrons. Attracted by a strong positive charge, the electrons in turn bombard the anode, which then produces X-rays. The X-rays penetrate the body, but their rate of transmission through different types of tissues depends on specific tissue attenuations. For example, bone attenuates X-rays more than air. Thus, a smaller amount of X-ray energy passes through bone, and its corresponding shadow on the developed radiographic film will be lighter. Conversely, the shadow of air will be darker. This explains why, on a chest X-ray, the ribs are relatively light, whereas the air containing lungs are relatively dark.

X-rays and gamma rays represent radiation with short wavelengths which is found at the higher energy end of the electromagnetic spectrum.

X-rays are produced when electrons strike the anode within a specially constructed vacuum tube.

X-rays penetrate air easier than they do soft tissue or bone. Thus, air produces a black image (exposes the film), and bone produces a white image (does not expose the film) on the X-ray film.

Conventional Tomography

Should we wish to see a particular plane within the human body, how can we achieve that with transmission radiography? If the X-ray tube, the patient, and the radiographic film remain stationary, we can create a series of shadows, many superimposed just like on a chest X-ray. However, if the patient remains stationary, but the X-ray tube and film move simultaneously (for the same distance, but in opposite directions), we will produce a tomogram (*tomos* means section or cut). Provided we place the fulcrum of the simultaneous motion of tube and film through the plane of interest in the human body, that particular plane will remain in focus and can be clearly visualized. Conversely, all tissues in front of and behind that plane will be out of focus, resulting in a blurring of their shadows. Thus, the tomogram represents an image of our plane of interest (see Figure 11-1B), unencumbered and unobscured by other tissues that happen to be within the same volume of tissue imaged but are of no interest to us.

Conventional tomography is the only cross-sectional method that does not depend on computers. All other modalities rely heavily on computer technology, especially in regard to image processing and formation. Further discussion needs to be prefaced by a few pertinent remarks about computers.

Analog and Digital Imaging

The computer (or digital processor) performs multiple functions, among others: image acquisition and control, image reconstruction, image storage and retrieval, and image analysis. Images must be in digital form before they can be processed by computer. Digital images consist of a matrix (usually a square, divided into rows and columns) in which each picture element (called a "pixel") is represented by a numerical value. A variable is said to be *analog* if it has a continuous value. On a chest X-ray, the shades of gray that compose the different shadows formed by tissues traversed by X-rays are theoretically infinite. They provide a smooth and continuous transition between maximum light (white) and maximum dark (black).

Computers are necessary for medical image acquisition, reconstruction, storage, and retrieval.

The smaller the pixel, the sharper the image.

Computed Tomography

Cross-sectional images are obtained by having the X-ray tube rotate around the patient. Opposite the tube, and on the other side of the patient, the transmitted X-rays are received, not by radiographic film but by an array of electronic detectors. Each detector will produce an electrical signal proportionate to the absorbed energy of the X-rays striking it. Later model CT machines have the tube sweep continuous 360° arcs around the patient, producing X-rays that are received by stationary detectors positioned along the entire circumference of the body. Every voxel (a volume element in a three-dimensional object that corresponds to one pixel in a two-dimensional image or picture) is thus sampled repeatedly (typically, hundreds of times) from many different angles.

Detectors are used to absorb energy from the X-ray to produce cross-sectional images.

The signals produced by the detectors are processed by a computer, which assigns a certain density value to the address of that voxel. *CT density*, which is analogous to lightness or darkness in film radiography, is expressed in Hounsfield units (HU) — after Godfrey Hounsfield, who was awarded the Nobel Prize in 1979 for his work on CT. By design, 1 HU represents a .1% difference in linear attenuation coefficient with respect to water. Water itself has been assigned an HU number of 0. Following is a sample of tissues

Density of CT image is described in Hounsfield units.

and their Hounsfield unit numbers: air: -1000, fat: 20 to -100, brain: 22 to 44, muscle: 44 to 59, liver: 50 to 80, bone: ~ 1000 HU. Clearly, CT provides much improved tissue characterization over plain film radiography (see Figure 11-2).

Single Photon Emission Computed Tomography (SPECT)

Nuclear medicine uses radioactive agents that are introduced into the body. These substances, also called *radio-pharmaceuticals* or *radionuclides* (see Chapter 13), are selected for their preferential uptake by certain organs or body compartments and their production of gamma rays that can be detected by specialized equipment. Alpha and beta particles are unsuitable for imaging, since they only travel a few millimeters or less in the body, while depositing potentially harmful doses of radiation. Ideally, radiopharmaceuticals need to emit gamma ray photons in the 70 to 500 keV range, which will likely escape the body, in order to be received by outside detectors. They should also have a half-life (the time necessary for half the radiopharmaceutical sample to decay) short enough to keep radiation absorbed by the body at a minimum, but not so short as to make imaging impossible. In SPECT, one or more detecting devices called *gamma cameras* slowly rotate around the patient's body. Gamma rays emitted from within the body hit specialized crystals in the gamma camera. Light signals are produced in crystals, which are ultimately processed by a computer to yield densities for specific detected and (small) areas on the final image. Anatomic detail is inferior to CT. Nuclear medicine (including SPECT) is primarily used to study the physiology of an organ or body part (see Figure 11-3). SPECT is good for imaging myocardial infarction (dead or dying heart muscle caused by significant decrease in blood supply) and a few other medical phenomena, such as the quantification of blood supply to the brain and for imaging certain bone fractures.

Radionuclides produce gamma rays that are detected by special scanners to produce an image of a body compartment or organ.

Images developed with nuclear medicine lack detail seen with CT scans but are useful to assess organ perfusion.

Electromagnetic energy is released from the collision of a positron and an electron.

Positron Emission Tomography (PET)

Certain elements contain nuclei that have a relatively high proton-to-neutron ratio. This may lead to instability of the atomic nucleus, resulting in the emission of a positron. This particle usually travels a very short distance before colliding with an electron. The two particles annihilate each other, and their masses convert into electromagnetic energy in the form of a pair of 511 keV photons. The two photons travel in nearly opposite directions (~ 180° to each other), and are received by detectors stationed on both sides of the patient. A coincidence circuit screens out

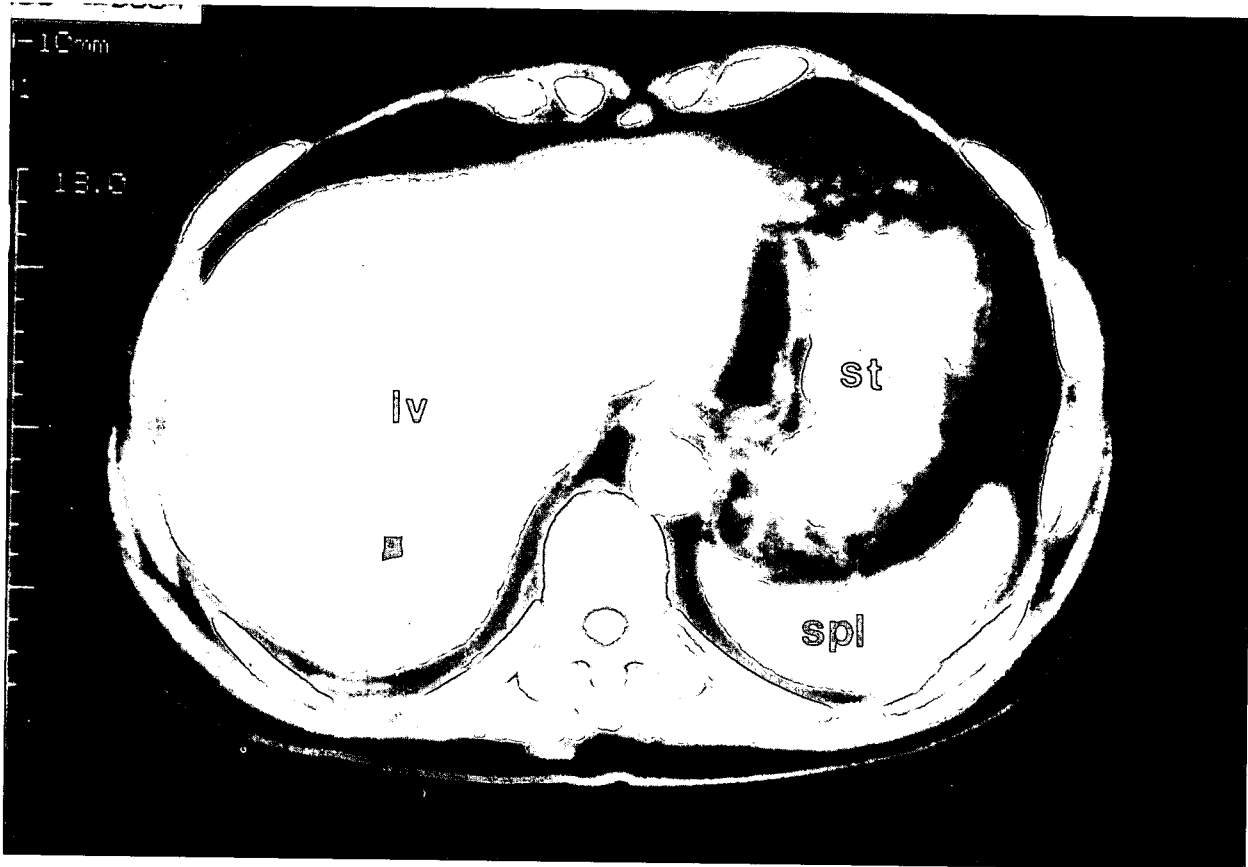


Figure 11-2

Computed tomographic scan of the upper abdomen showing the liver (lv), spleen (spl), and stomach (st).

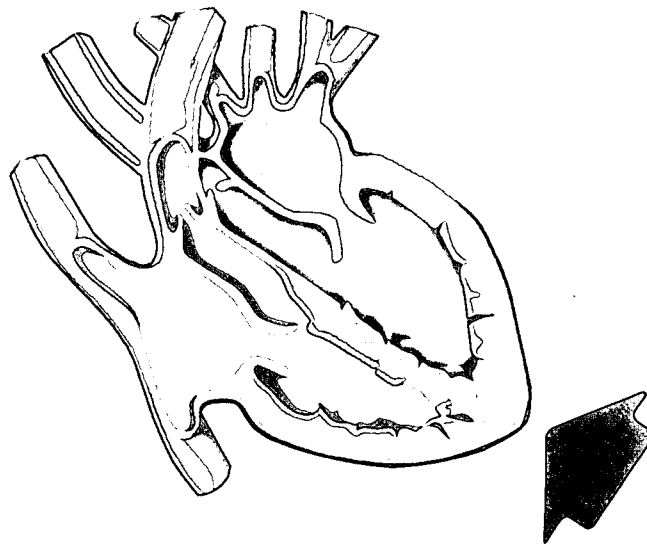
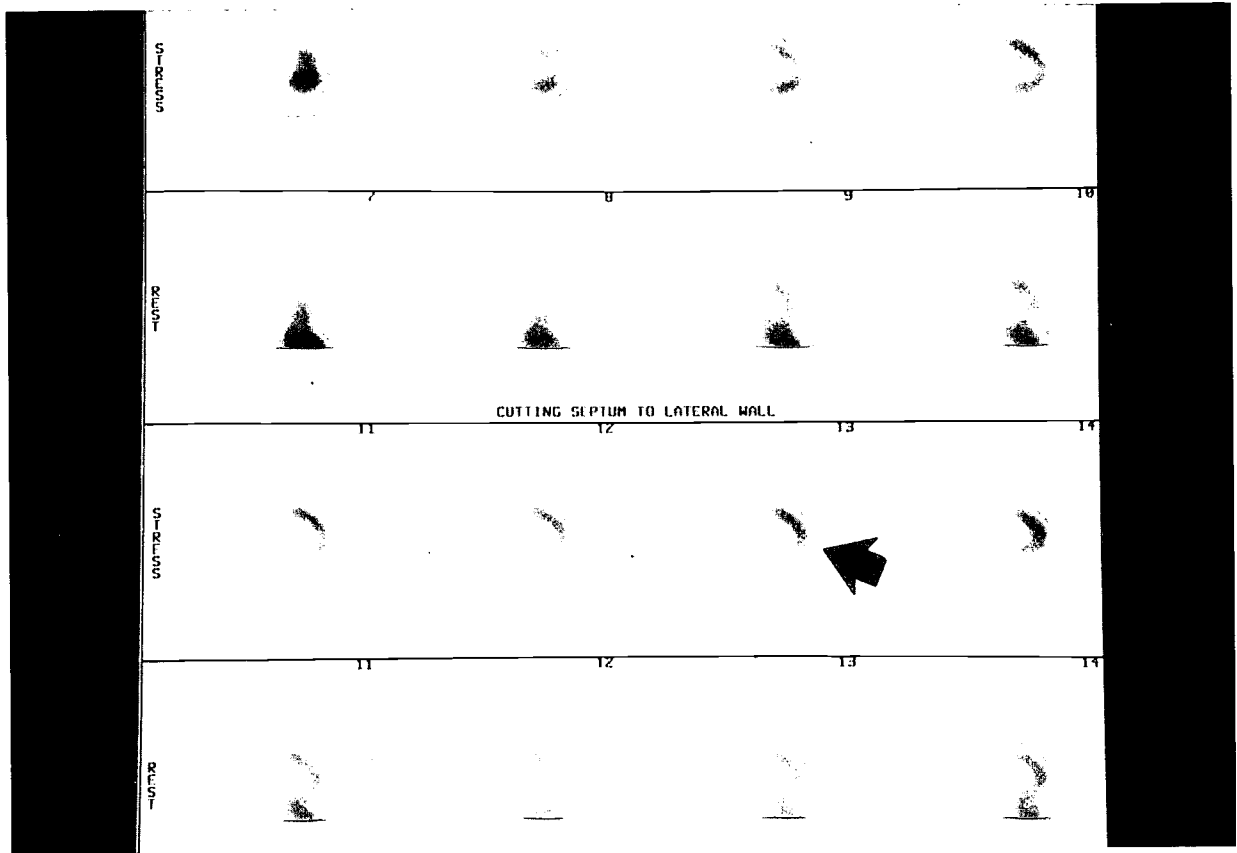


Figure 11-3
A SPECT image of the myocardium (heart muscle).
(Cartoon used as an aid to identify structure)

PET images physiologic function of body organs such as the myocardium.

everything but signals received by two opposing detectors struck almost simultaneously by these photons. PET does not lend itself well to anatomic imaging but can be used to study subtle physiologic processes not otherwise detectable by other imaging methods. Only a small number of PET centers operate in the United States and abroad, the main constraint being the high cost of the technology. This is, in part, caused by the very short half-lives of many PET radionuclides, typically ranging from a few minutes to 1 or 2 hours. This constraint virtually dictates that a cyclotron (a machine that produces these short-lived radiopharmaceuticals) be present on site.

Ultrasonography

Ultrasound uses sound at a frequency range of 1-15 MHZ to generate an image.

During World War II, a new detection system (SONAR, an acronym for *Sound Navigation and Ranging*) of underwater objects was successfully developed and used by the United States Navy. An offshoot of this subsequently became useful in medical imaging and is called *ultrasonography*. Where audible sound has a frequency range of 20-20,000 Hz (hertz, or cycles/second), ultrasound uses frequencies of 1 to 15 MHZ (1 MHZ= 1 million Hz), well outside human hearing range. Sound (and ultrasound) causes waves of compression and rarefaction to propagate through a medium (gas, liquid, soft tissue, etc.). Sound is similar to X-rays in that it transfers energy from one point to another, but it is not a form of electromagnetic radiation. This difference means that sound passes only through matter and cannot traverse a vacuum.

Ultrasound waves pass through tissue and fluid, but not air and bone.

When the ultrasound beam passes through different tissues, sound energy is reflected at the interfaces between them. The time it takes for this echo to return is proportional to the depth of the interface. The intensity of the echo is determined by the different physical properties of the organs or regions on both sides of the interface and on its depth. Electrical pulses are used to create ultrasound waves. The device that transmits the ultrasound waves and simultaneously records the reflected echo is called a *transducer*. The reflected echoes are converted into electrical pulse and fed into a computer. The transducer must be kept in close contact with the patient's body. Contact between the transducer and the body surface can be enhanced with the use of a gel. The computer collects the data and forms structural images. Ultrasound does not yield the high degree of anatomic detail obtained with use of CT scans or MRI.

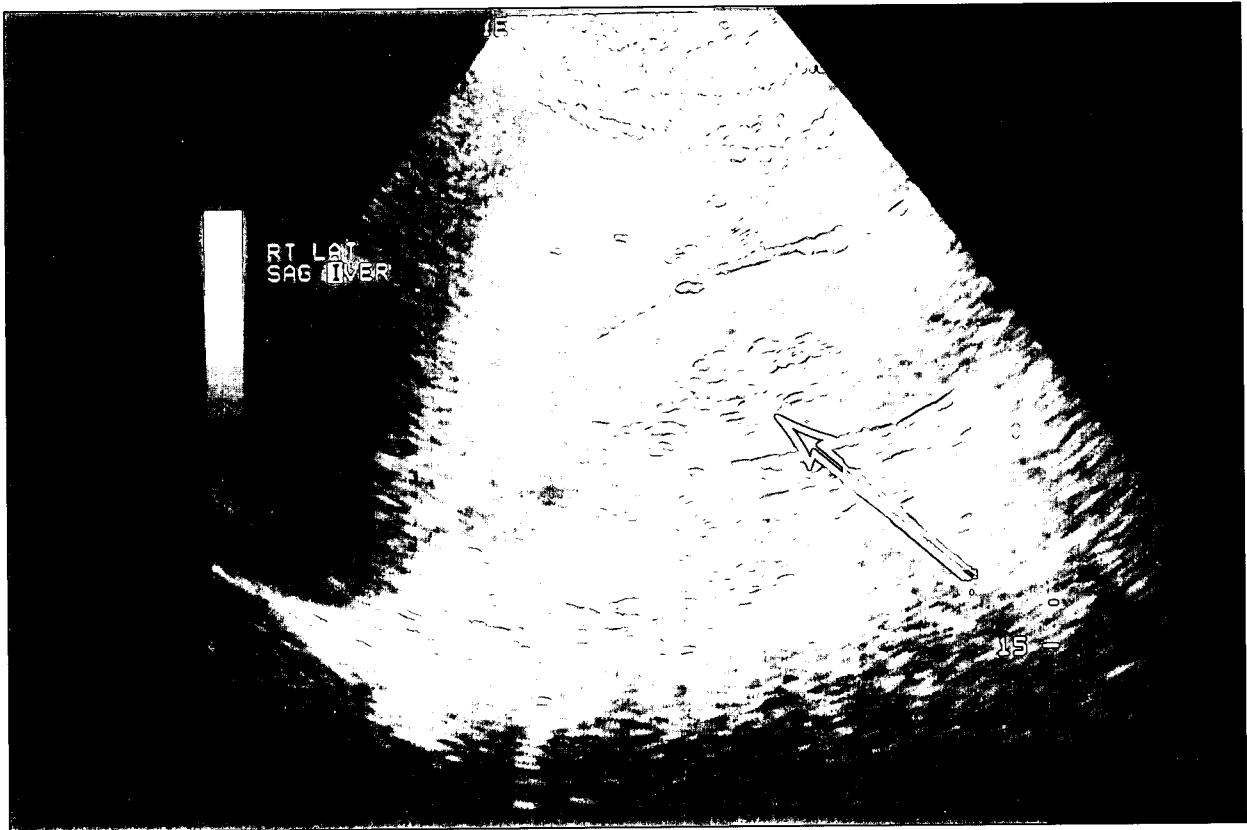


Figure 11-4
Ultrasound image of a human kidney.

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Ultrasound also does not penetrate air (gases) or bone. Despite these limitations, it is very popular since it does not use ionizing radiation (and can thus be used to safely examine pregnant women or the eyes, for example). The equipment required is less expensive than CT or MR scanners, and portable.

Magnetic Resonance Imaging (MRI)

When a proton is placed in a magnetic field, it aligns with the axis of the field. When the proton is pushed out of alignment, its angle of precession (spinning around its axis) increases. The proton then goes through the process of re-alignment, during which a tiny amount of energy is released. When a patient lies within the hollow bore of a gigantic magnet, his or her hydrogen nuclei (protons) behave similarly. In MRI, radio frequency pulses are used to knock the protons out of alignment. The tiny packets of energy released are received by detectors, and since they carry information about the spatial location of a specific tissue within the body, a computer can create the image through some very complicated physics calculations. The brightness of each pixel in the image depends on the intensity of the radio frequency signal originating from the corresponding tissue voxel. In turn, the signal intensity of a voxel is determined by nuclear density, longitudinal relaxation rate (or T1), transverse relaxation rate (or T2), and flow. It is possible to perform the procedure in such a way as to produce images that are "weighted" more or less heavily in favor of certain of these factors. Thus, in the most commonly used MRI technique, the *spin-echo sequence*, images can be created that are T1 or T2 weighted. Water is hypointense ("dark") on T1, whereas fat is hyperintense ("bright"). It is the opposite on T2 weighted images. Many other substances have different, or similar, signal intensities on T1 and T2 weighted images. Combining the information from these different images helps in determining if a mass contains fluid or fat, or if a blood clot is new or old, for example. The advantages of MRI are its superior anatomical delineation, its ability to depict many physiologic and pathologic processes, and its absence of ionizing radiation. It also allows direct multiplanar imaging; that is, any plane can be imaged without having to reposition the patient. This is different from CT, where the patient needs to be repositioned every time, and where certain planes are impossible to image due to physical limitations. Disadvantages of MRI are its high cost and length of the procedure, thus making it difficult to use with very sick or uncooperative

MRI creates superior anatomic images that can be used to assess physiologic and pathologic processes without the use of radiation.

patients. Since the magnetic field can make certain metal objects move, people with aneurysm clips in the brain, foreign bodies in the eye, or prostheses in the ear, should not have MRI unless there is certainty that these metal objects will not be made to move by the magnet. Since MRI signals may cause electronic interference, people with pacemakers are also excluded.

Image Interpretation

No imaging of any sort should be performed unless indicated by the patient's condition. The choice of imaging modality should be determined by what can best find and picture the problem with the least hazard and discomfort to the patient. Availability of the equipment and cost are other factors to be considered. Once the study is done and the images obtained, interpretation should be performed by trained personnel, in most cases *radiologists*. A solid understanding of normal anatomy and physiology is a must, as is knowledge of the different abnormal imaging patterns often indicating pathologic causes. These may occur because an organ or an area in the body has changed in size, shape, internal architecture, or a combination of those factors — or because the abnormal area is “darker” or “lighter”, has more echoes or fewer, is hyperintense, or hypointense; becomes “hotter” by taking up more of a radiopharmaceutical or “colder,” etc. Interpretation is often helped if contrast material is injected or otherwise introduced into the patient. CT and MRI of the brain, for example, often show abnormalities better when contrast is enhanced. Technological innovations and advances were responsible for the origin and growth of radiology and the imaging sciences. Future developments and discoveries will undoubtedly provide for better, safer, and faster imaging modalities and significant improvements in existing ones. We may not have seen anything yet, since with the exception of Roentgen's discovery, the major innovations have only taken place in the last few decades. We would also hope that imaging technologies will become cheaper or at least come with a less expensive initial price tag, but previous experience has not proved this to be the case.

For accurate interpretation of any radiologic image, a good understanding of anatomy and physiology is necessary.

Summary

Imaging of the human body can be done by transmission of X-rays (such as with chest films, or spinal films). Cross-sectional images can also be obtained, using a variety of means,

using ionizing radiation, a magnetic field and radio frequency pulses, and ultrasound waves. Most of these modalities have been made possible by progressive advances in computer technology. Each method has its advantages, disadvantages and limitations. The choice of imaging procedure must be dictated by what is best for the patient, with other factors such as availability and cost playing important roles as well.

Questions Commonly Asked by Students

Question 1: What is the best imaging study, should I ever need one?

That will pretty much be dictated by the nature of your medical condition. If you are perfectly healthy, but your prospective college or employer wants you to get a complete physical check-up, you will very likely be required to have a chest X-ray. This is a standard radiograph, not a cross-sectional image, and it is adequate for that purpose. However, if the chest X-ray shows a lung nodule which needs further investigation, you will probably require a CT scan of your chest.

CT is also excellent for examination of the abdomen and pelvis. MRI is superior for depiction of the central nervous system, although CT is preferred in acute trauma situations such as in motor vehicle accidents. MRI is also useful for the heart and musculoskeletal system and is often used in conjunction with CT. SPECT and PET are performed when disturbances of physiology are suspected, rather than of anatomy, for which CT and MRI are superior.

US is preferred in many conditions because of its ease and its absence of ionizing radiation. Investigation of the abdomen, pelvis and obstetrical conditions, for example, is often done with US. Conventional tomography is seldom used today, its function having been superseded by the other methods, especially CT.

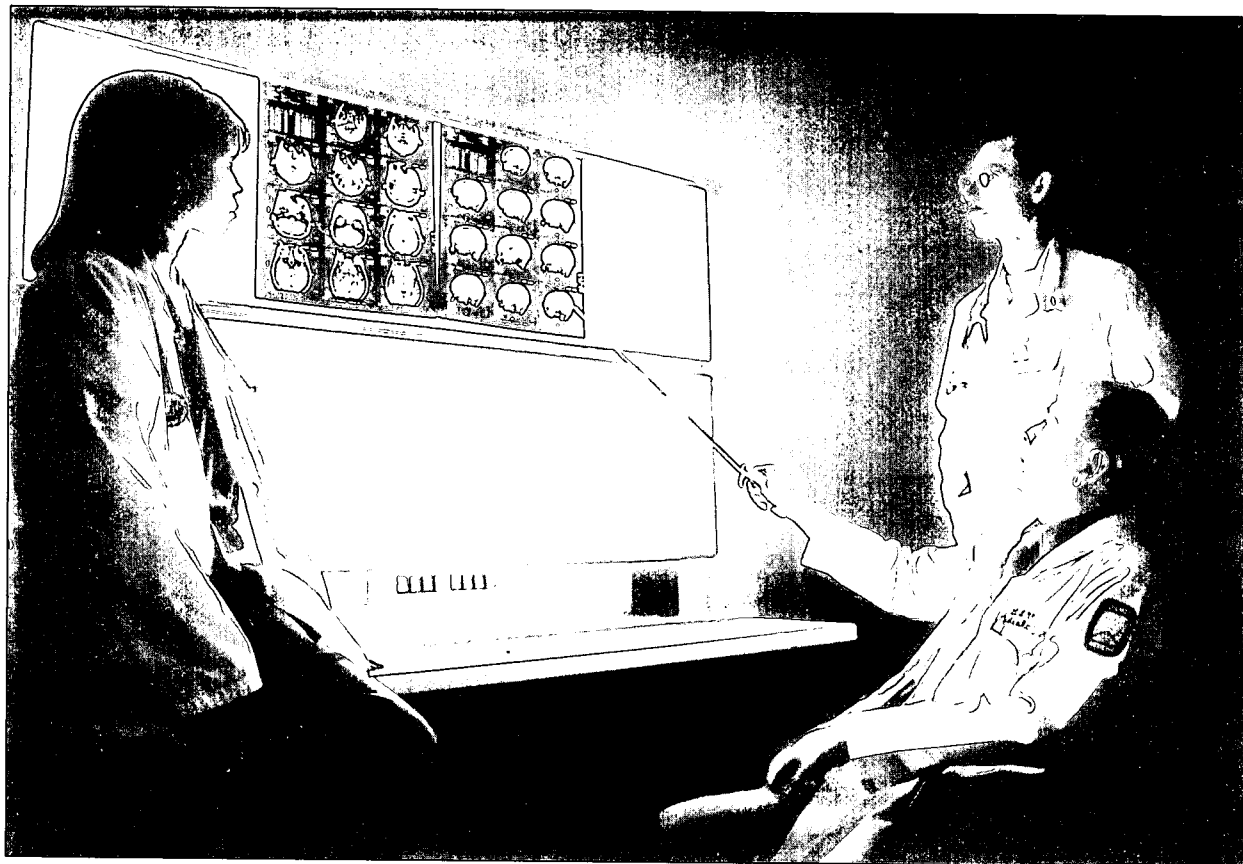
Question 2: It seems like these cross-sectional imaging techniques may be potentially dangerous. Which method is the safest?

It is generally, but not universally, agreed that the low doses of radiation diagnostic that imaging equipment produce are not harmful to human beings. Of course there are certain precautions to be taken. Pregnant women should not be exposed to ionizing radiation, especially during the first trimester, when the fetus is most susceptible.

People with certain foreign or surgical objects in their body may not be suitable for MRI. One should always consider alternatives, but the difficult decision may ultimately have to be made as to whether the possible information to be gained by the imaging method warrants the risk. Protective measures can minimize exposure. For example. CT scan of the brain of a pregnant woman is done with adequate lead shielding of her abdomen and pelvis.

Question 3: I hear that these imaging techniques can be very expensive. Which is the cheapest?

It is true that many of these marvels of technology come with hefty price tags. However, they often allow physicians to arrive at a more accurate diagnosis faster, thus saving time, avoiding other — and probably more lengthy — costly, and potentially more painful and dangerous investigative procedures. It is almost paradoxical that these expensive cross-sectional techniques actually save healthcare dollars, especially when used wisely. As a general statement, US is least expensive, and PET is the most expensive. CT falls toward the lower end of the price scale, with MRI and SPECT toward the upper end.



Dr. M. Hardjasudarma (right) explains interpretation of a computed tomogram image (CAT scan) of the head to Natalie Adams (left), a senior student in the Cardiopulmonary Science Program at LSU Medical Center, School of Allied Health Professions, and Jeff Lantz, BS, RRT, who is the Patient Care Coordinator (center) for the Department of Cardiopulmonary Services at the LSU Medical Center Hospital, Shreveport, LA.

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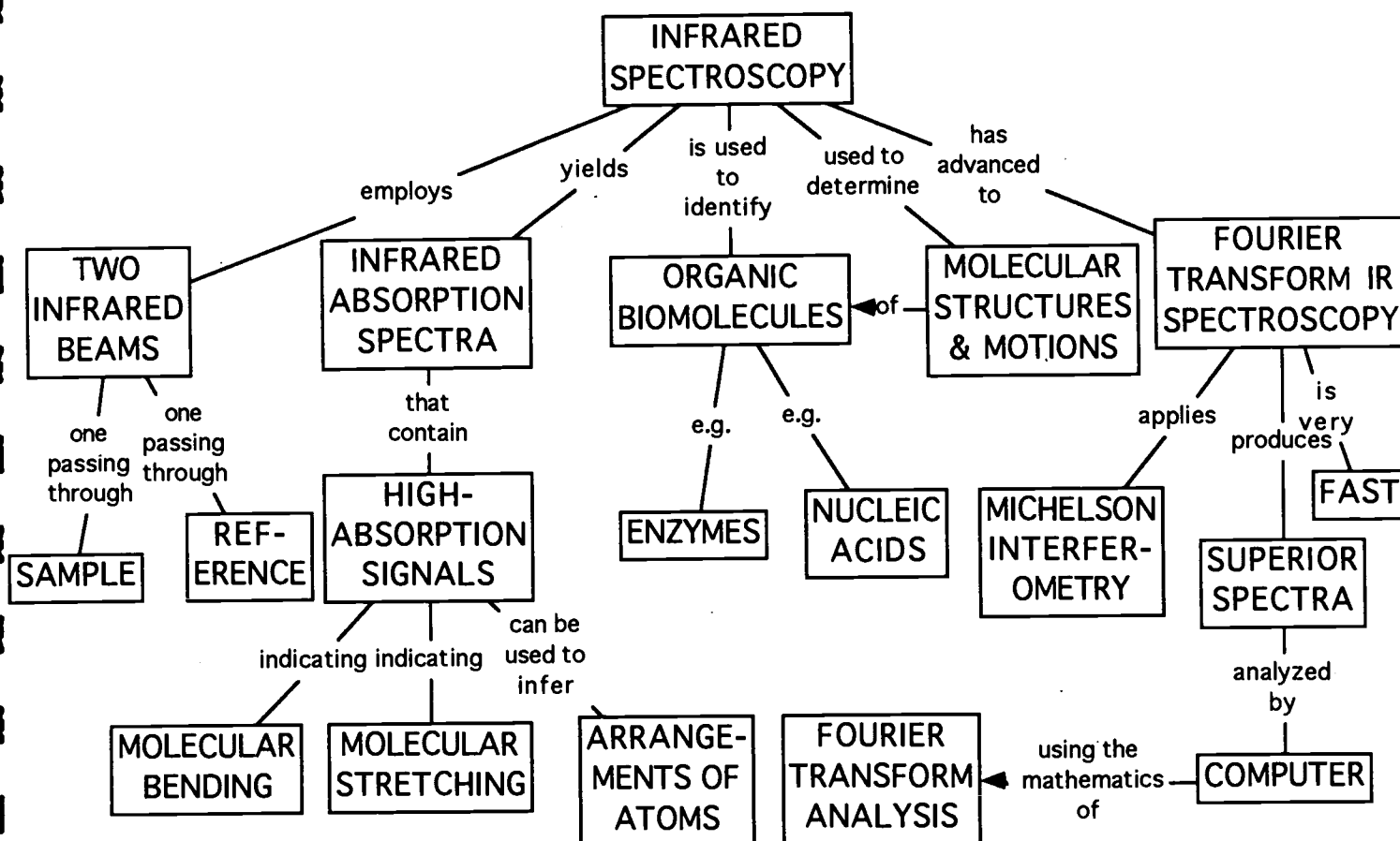
INTRODUCTION TO INFRARED SPECTROSCOPY

12

Gary Lyon

Overview

1. Nature of electromagnetic radiation
2. Interpretation of infrared spectra
3. Types of sample holders
4. Types of infrared spectrometers
5. Applications of infrared spectrometry



An Introduction to Infrared Spectroscopy

Light is a form of radiant energy, and it has wave properties.

Visible light, or light energy that can be detected by the human eye, is one form of electromagnetic radiation. Other common forms of electromagnetic radiation include infrared radiation, radio waves, microwaves, ultraviolet light, X-rays, and gamma rays. Spectroscopic methods for the identification and characterization of chemical compounds, including infrared, visible, and ultraviolet spectroscopy, depend upon the interaction of electromagnetic radiation ("light") with different kinds of molecules. *Infrared spectroscopy* is particularly useful for the characterization of both organic and biomolecules because it yields information regarding the presence of various organic functional groups.

The Nature of Electromagnetic Radiation

The distance between the same point on successive waves is called the wavelength of that light.

Classically, light and other forms of electromagnetic radiation are considered to be waves that can be characterized by both their wavelength and frequency. *Wavelength* (see Figure 12-1A) may be measured in any convenient units; however, infrared spectroscopists usually measure wavelengths in centimeters (cm). *Frequency* is defined as the number of waves passing a given point in a given time, and has units of (time⁻¹), usually s⁻¹ ("reciprocal seconds"). The frequency (ν) and wavelength (λ) are related using the equation $\nu\lambda = c$, in which c is the speed of light (3.00×10^{10} cm/s). In other words, when the wavelength is small, the frequency is large; when the wavelength is large, the frequency is small.

The number of light waves passing a given point in a given time is referred to as the frequency of that light.

Light and other radiant energies are characterized by both wavelength and frequency.

The human eye can detect electromagnetic radiation (light) having wavelengths between about 4.0×10^{-5} cm and 7.0×10^{-5} cm (see Figure 12-1 B). In contrast, infrared radiation has a longer wavelength; thus, it cannot be detected by the human eye. Most spectroscopic measurements in the infrared region are made using wavelengths between 2.5×10^{-4} cm and 5.0×10^{-3} cm. These numbers are usually expressed using another quantity, the *wavenumber*, which is defined as the inverse of the wavelength ($1/\lambda$). Therefore, the region of infrared radiation commonly studied by spectroscopists has wavenumbers between $4,000 \text{ cm}^{-1}$ and 200 cm^{-1} ("reciprocal centimeters"). Large wavenumbers are commonly found on the left side of the infrared spectrum, and small wavenumbers on the right.

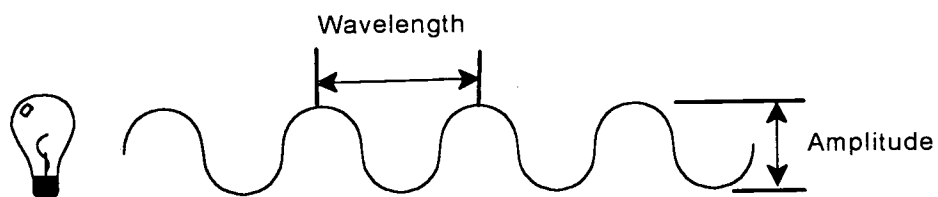


Figure 12-1A

A wave of light. Wavelength (λ) is measured crest-to-crest. The wavenumber is the inverse of the wavelength.

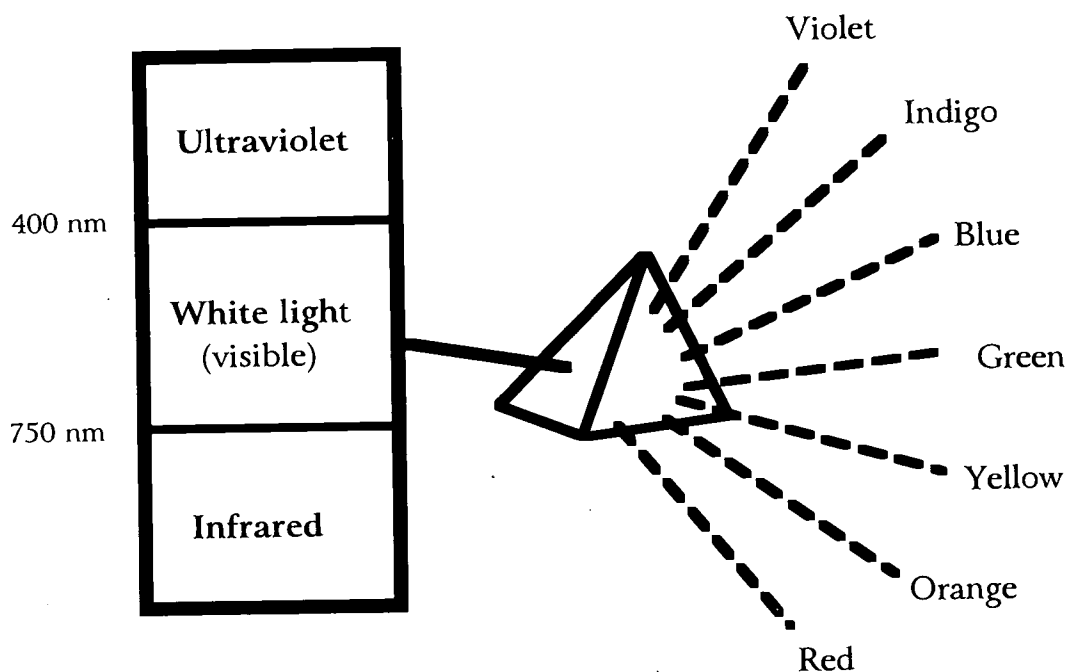


Figure 12-1B

Refraction of white light through a prism.

Radiant energy exhibits both wave-like and particle-like behavior.

Photons are particles or packets of light energy.

Absorption of photons of electromagnetic radiation by molecules results from molecular rotation, vibration, or electronic transitions within molecules.

Infrared spectroscopy measures vibrations that occur within molecules that absorb infrared radiation.

Transmittance refers to the measurement of the amount of light that passes through a substance and is expressed as a percentage.

Described in modern quantum-mechanical terms, electromagnetic radiation has both a wave-like and particle-like nature and is sometimes described as a wave-packet or *photon*. The energy of a photon is a function of its wavelength. Photons of high-energy electromagnetic radiation have a high frequency and a short wavelength; those of low energy electromagnetic radiation have a low frequency and long wavelength. Approximate wavelengths of various forms of electromagnetic radiation are given in Table 12-1.

The absorption of photons of electromagnetic radiation by molecules can generally be attributed to one or more of three causes: (1) rotation of molecules, (2) vibration of molecules, and (3) electronic transitions within molecules. Rotation of molecules occurs when low-energy radiation (e.g., microwaves) is absorbed. When food is placed in a microwave oven, the low-energy microwaves are absorbed by water molecules, causing them to rotate and increasing the temperature of the food. Vibrations within molecules (sometimes accompanied by rotations) occur when low-energy (infrared) radiation is absorbed. These are the molecular motions studied by the infrared spectroscopist. Finally, electronic transitions occur when molecules absorb higher energy visible and ultraviolet light. Colored compounds such as dyes and pigments exhibit electronic transitions when they absorb visible light.

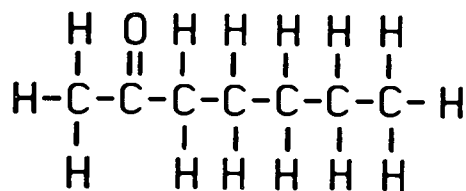
The Interpretation of Infrared Spectra

Infrared spectroscopy is, therefore, the technique used to study the vibrations that occur within molecules when infrared radiation is absorbed. The instrument used to study and record this absorption is the infrared spectrometer, and the output is an infrared spectrum. Figure 12-2 and Figure 12-3 show the structure and infrared spectrum of 2-heptanone. Percent transmittance (a measure of the amount of infrared radiation which passes through the sample) is plotted on the ordinate (y-axis) of the spectrum, and wavenumber on the abscissa (x-axis). Wavenumbers of higher energy (closest to visible light) occur on the far left, while wavenumbers of lower energy occur toward the right. A signal is detected when the percent transmittance (%T) is low. This indicates that the molecule has absorbed infrared radiation at a specific wavenumber. One example of a signal is the sharp dip in the spectrum of 2-heptanone which occurs at 1700 cm^{-1} (see Figure 12-3). Some signals indicate the presence of certain functional groups, or arrangements of atoms,

Table 12-1. Approximate Wavelengths of Some Forms of Electromagnetic Radiation.

Type of Energy	Wavelength (cm)	Energy
Radio Waves	c. 10^{-2}	
Microwaves	c. 1	very low
Infrared radiation	c. 10^{-3}	low
Visible light	$< 10^{-4}$	moderate
Ultraviolet light	c. 10^{-5}	moderate
X-rays	c. 10^{-7}	high
Gamma rays	c. 10^{-10}	very high

A



B

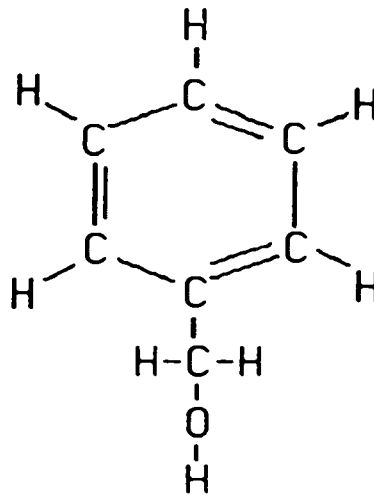
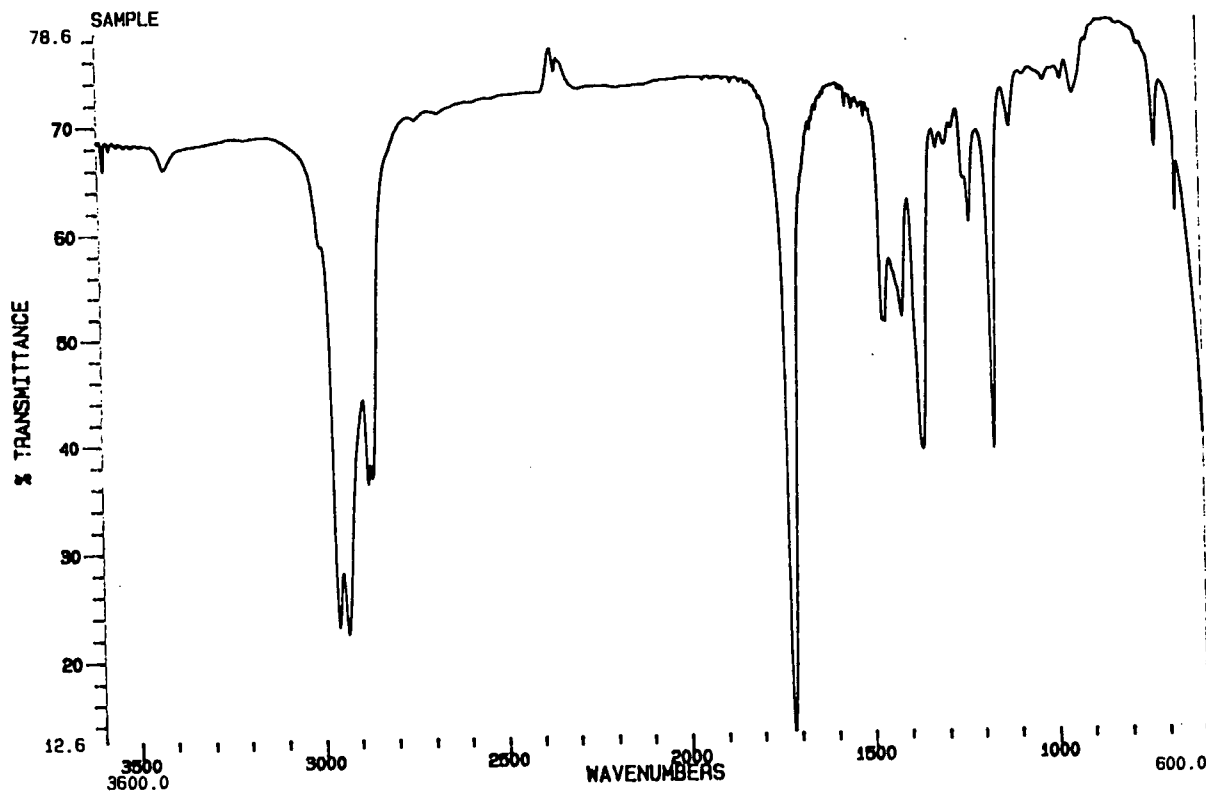


Figure 12-2
Structures of 2-heptanone (A) and benzyl alcohol (B).



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Figure 12-3
Infrared spectrum of 2-heptanone.

Using percentage transmittance, infrared spectroscopy can identify chemical composition.

When a photon of light is absorbed and molecular stretching or bending occurs, a signal is recorded in the infrared spectrum.

Each chemical compound has a unique pattern of stretching or bending signals.

in the molecules being studied. So, the infrared spectrum can be used as a tool in the identification of chemical compounds.

Two common forms of vibrational motion are the stretching and bending of atoms within molecules (see Figure 12-4). An analogy of stretching within a molecule is the stretching that occurs when two or more balls are connected using springs. If a molecule consists of more than two atoms, different types of stretching are possible and are described using the terms *symmetric stretching* and *asymmetric stretching* (see Figure 12-4). Many different types of bending are possible within molecules, and these bending motions are sometimes described using terms such as *scissoring*, *twisting*, *wagging*, and *rocking* (see Figure 12-4). When a photon of light is absorbed and stretching or bending occurs, a signal is recorded in the infrared spectrum. Because stretching generally requires more energy than bending, infrared signals indicating stretching generally occur at higher wavenumbers (on the left side of the spectrum). Signals caused by bending motions within molecules are frequently more numerous and more difficult to interpret than those caused by stretching, and it is therefore generally much easier to interpret the signals on the left side (those caused by stretching) first. Although it is usually difficult to interpret all of the bending signals in an infrared spectrum, each compound gives a unique pattern of stretching and bending signals. The region of the infrared spectrum in which bending patterns occur is frequently called the *fingerprinth region*. A few of the most common stretching signals and their approximate wavenumbers are listed in Table 12-2.

The molecular structure of 2-heptanone is given in Figure 12-2 A. Comparison of the infrared (IR) spectrum of this compound (see Figure 12-3) with the signals listed in Table 12-2 gives the following information: (a) Signals indicating the O-H stretch and the N-H stretch in the vicinity of $3600 - 3200 \text{ cm}^{-1}$ are absent, indicating that the compound is neither an alcohol, an amine, nor an amide; (b) the strong C=O stretching signal at 1700 cm^{-1} is characteristic of aldehydes and ketones; and (c) the presence of several strong C-H stretching signals between 3000 and 2850 cm^{-1} coupled with the absence of any signals between 3100 and 3000 cm^{-1} is characteristic of compounds containing no carbon-carbon double bonds. All of these features are consistent with the structure of 2-heptanone.

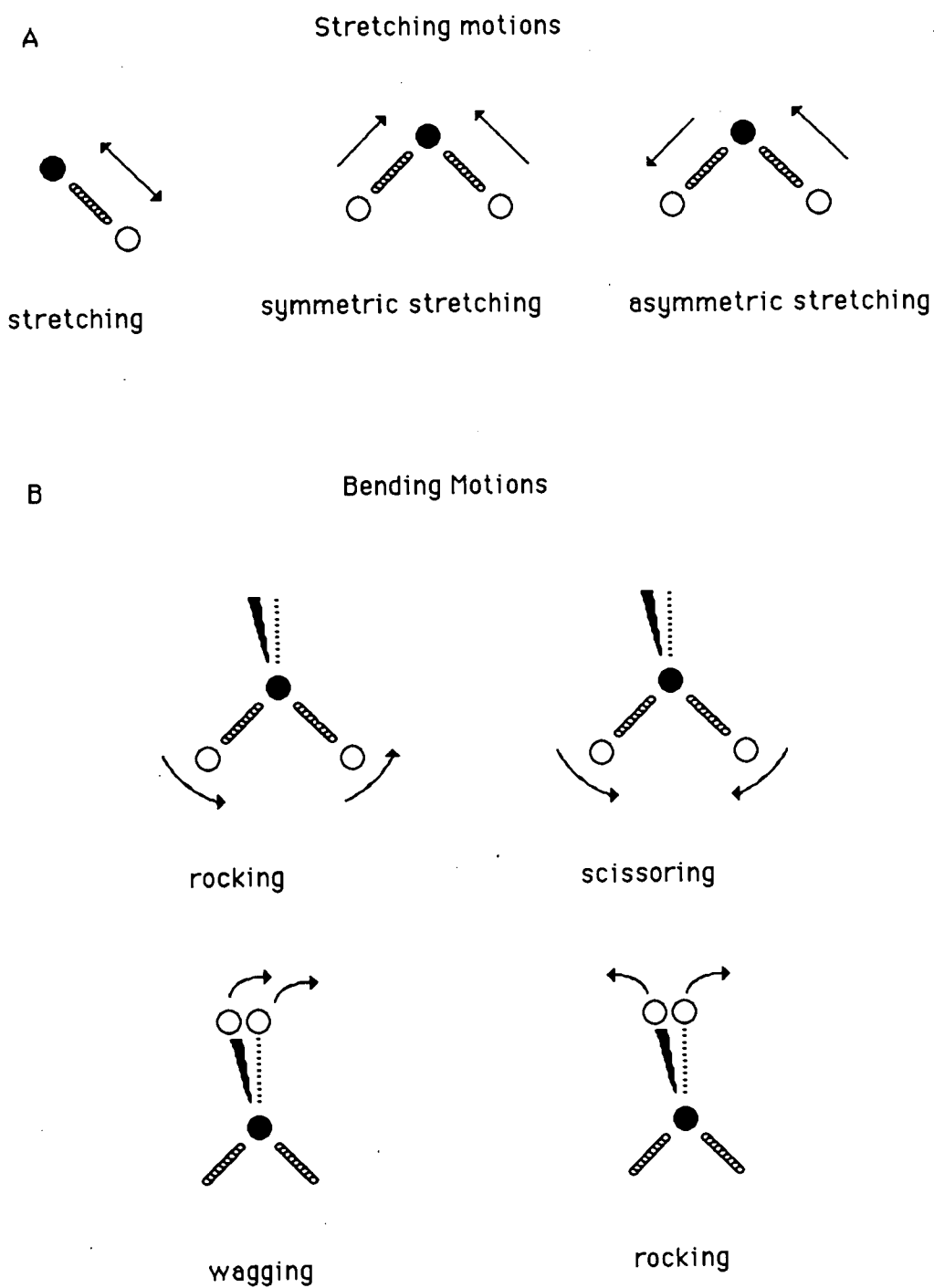


Figure 12-4
Types of stretching and bending motions within molecules.

Table 12-2. Common Infrared Stretching Signals.

Type of Signal	Wavenumber (cm^{-1})	Band Characteristics
C-H stretch		
sp^2 hybridized C	3100-3000	
sp^3 hybridized C	3000-2840	
- CH_3 symmetric stretch	2872 cm^{-1}	
- CH_3 asymmetric stretch	2962 cm^{-1}	
O-H stretch		
alcohols	3700-3350 cm^{-1}	strong, broad
carboxylic acids	3300-2500 cm^{-1}	very broad
C=C stretch		
alkenes	1650-1600 cm^{-1}	weak
aromatics (benzene and derivatives)	1610-1480 cm^{-1}	moderate
C=O stretch		
aldehydes, ketones, carboxylic acids	1850-1500 cm^{-1}	
amides	1750-1700 cm^{-1}	strong, sharp
amides	1650 cm^{-1} (amide I band)	
N-H stretch		
primary, secondary amines, amides	near 3500 cm^{-1}	moderate, sharp

Signals found on a compound's IR spectra can be used to infer its structure.

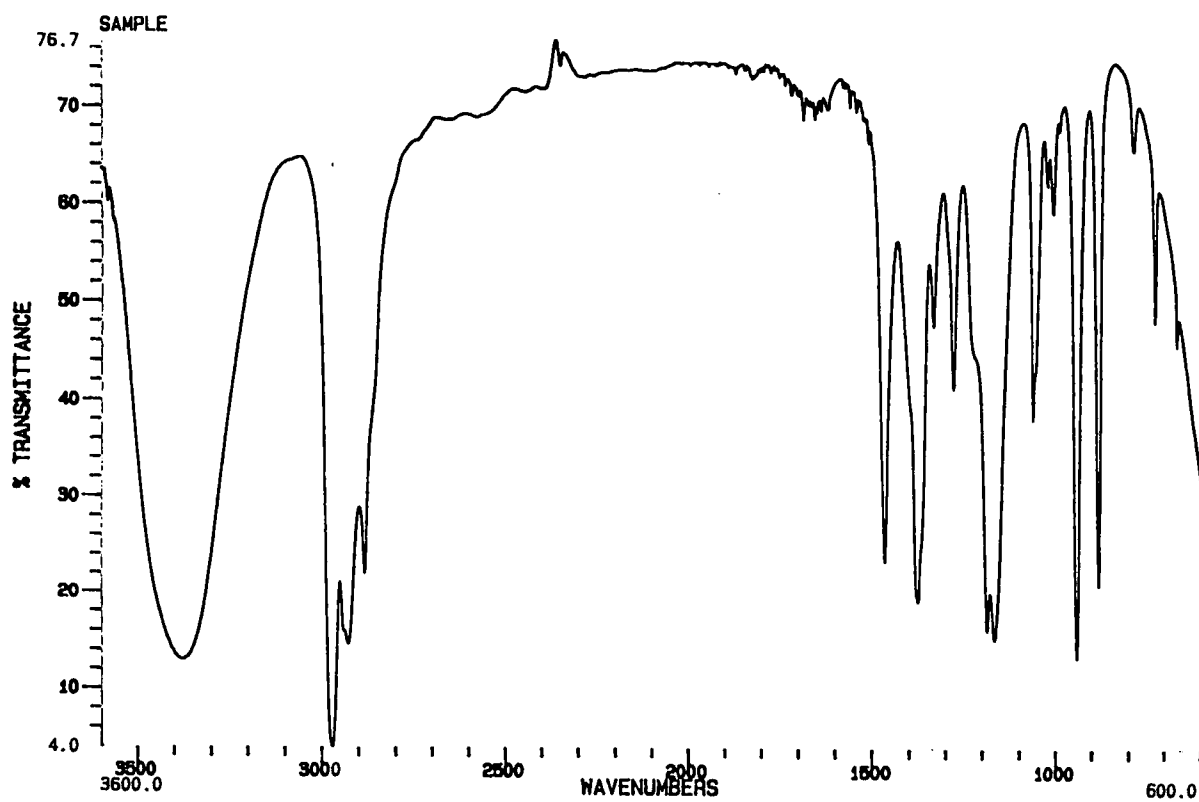
The structure of benzyl alcohol is given in Figure 12-2 B. Comparison of the spectrum of benzyl alcohol (see Figure 12-5) with that of 2-heptanone reveals the following differences: (a) the spectrum of benzyl alcohol has a strong, broad signal in the area characteristic of the O-H stretch (3700 to 3350 cm^{-1}), (b) the carbonyl stretching signal (c. 1700 cm^{-1}) characteristic of aldehydes and ketones is absent, and (c) there are several signals, both immediately above and immediately below $3,000$ cm^{-1} , characteristic of the C-H stretch associated with hydrogen atoms attached to both sp^2 -hybridized carbons (the carbon atoms in the benzene ring) and sp^3 -hybridized carbon (the carbon atom bearing the -OH functional group).

Types of Sample Holders

Infrared spectroscopy can be applied to solids, liquids, or gases.

Samples to be investigated by infrared spectroscopy may be either solids, liquids, or gases. Sample preparation for non-aqueous liquid samples is fairly straightforward. A thin film of the liquid is sandwiched between two plates of polished crystals (*salt plates*) commonly made of sodium chloride. Sodium chloride is used because the sample holder must be transparent to infrared radiation; therefore, ordinary window glass, quartz, and various plastics are all unacceptable. Because salt plates are water soluble, they must be handled with care. Aqueous solutions, commonly encountered in many biological systems, would react with sodium chloride salt plates. However, aqueous solutions may be placed between crystals of silver chloride (AgCl), which is water insoluble and is also transparent to most wavelengths of infrared radiation.

Solid samples can be finely ground with powdered potassium bromide and formed into transparent pellets using a high-pressure pellet press. Some solid samples are transparent polymer films. These can be mounted in the spectrometer without any further preparation. Special cells have been developed for the determination of gaseous samples. Because fewer gas molecules exist in a given volume, gas samples must be thicker than liquid or solid samples, and the gas cell commonly has a length of about 10 cm. Most gas cells are cylindrical and have polished sodium chloride disks at each end. A gas cell is filled by evacuating it and then connecting it to the gas source.



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Figure 12-5
Infrared spectrum of benzyl alcohol

Types of Infrared Spectrometers

Although the components are different, a traditional infrared spectrometer (see Figure 12-6) is conceptually similar to the more common type of spectrometer which detects visible light. The radiation source is either a Nernst filament (see Figure 12-6A), composed of oxides of zirconium, thorium, and cerium, or a Globar filament made of silicon carbide. Both sources emit infrared radiation when heated to temperatures between 1000° C and 1800° C. The infrared radiation is focused and split into two beams by parabolic mirrors (see Figure 12-6B) commonly made of polished aluminum. One beam passes through the sample (see Figure 12-6C), and the other passes through a reference (see Figure 12-6D). If the sample is pure, no reference is needed; otherwise, the reference commonly consists of the solvent used to dissolve the sample. In biological systems, this solvent is usually water.

Two infrared beams, one passing through the sample and the other through a reference, are needed in the infrared spectrometer.

The sample beam and reference beam then pass through another mirror system (see Figure 12-6E, the "chopper") which produces one beam (rapidly alternating between the sample and reference). This beam then passes through a diffraction grating (see Figure 12-6F, the monochromator), which splits the beam into its component wavelengths in a manner somewhat analogous to the splitting of visible light into various colors by a prism. As the monochromator rotates, different wavelengths reach the detector (see Figure 12-6G). The detector then sends an electrical signal to a strip chart recorder, which produces spectra similar to those shown in Figures 12-3 and 12-5.

Instruments such as this, known as *grating* or *diffraction spectrometers*, have been shown to be rugged and reliable, and continue to give excellent service. However, recent advances in computer technology have made the *Fourier transform infrared spectrometer* (FTIR, see Figures 12-7 and 12-8) the instrument of choice in many laboratories. The source of the FTIR spectrometer (Figure 12-7A) emits infrared radiation which is focused by mirrors (Figure 12-7B) and split into two perpendicular beams by the beam splitter (Figure 12-7C). One beam, of fixed length, is reflected by a fixed mirror (Figure 12-7D); and the other beam is reflected from a movable mirror (Figure 12-7E). This second beam varies slightly in length. These two beams of radiation are then recombined to form a single modulated beam which is relatively rich in certain wavelengths of radiation depending on the position of the movable mirror. This type of

Fourier transform instruments are useful in measuring biological samples that degrade rapidly, and these instruments are also capable of detecting weak signals.

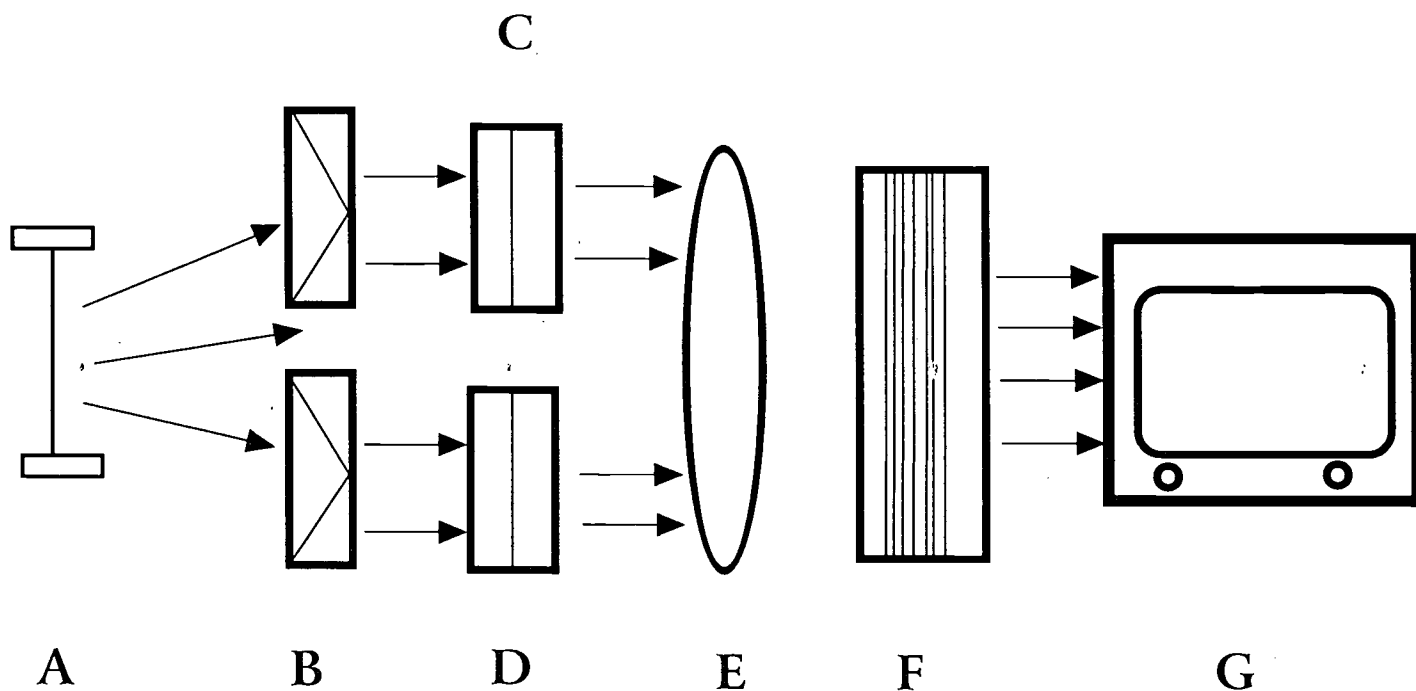


Figure 12-6

Schematic diagram of an infrared spectrometer. A, source; B, parabolic mirrors (optics); C, sample; D, reference; E, chopper; F, monochromator; G, detector.

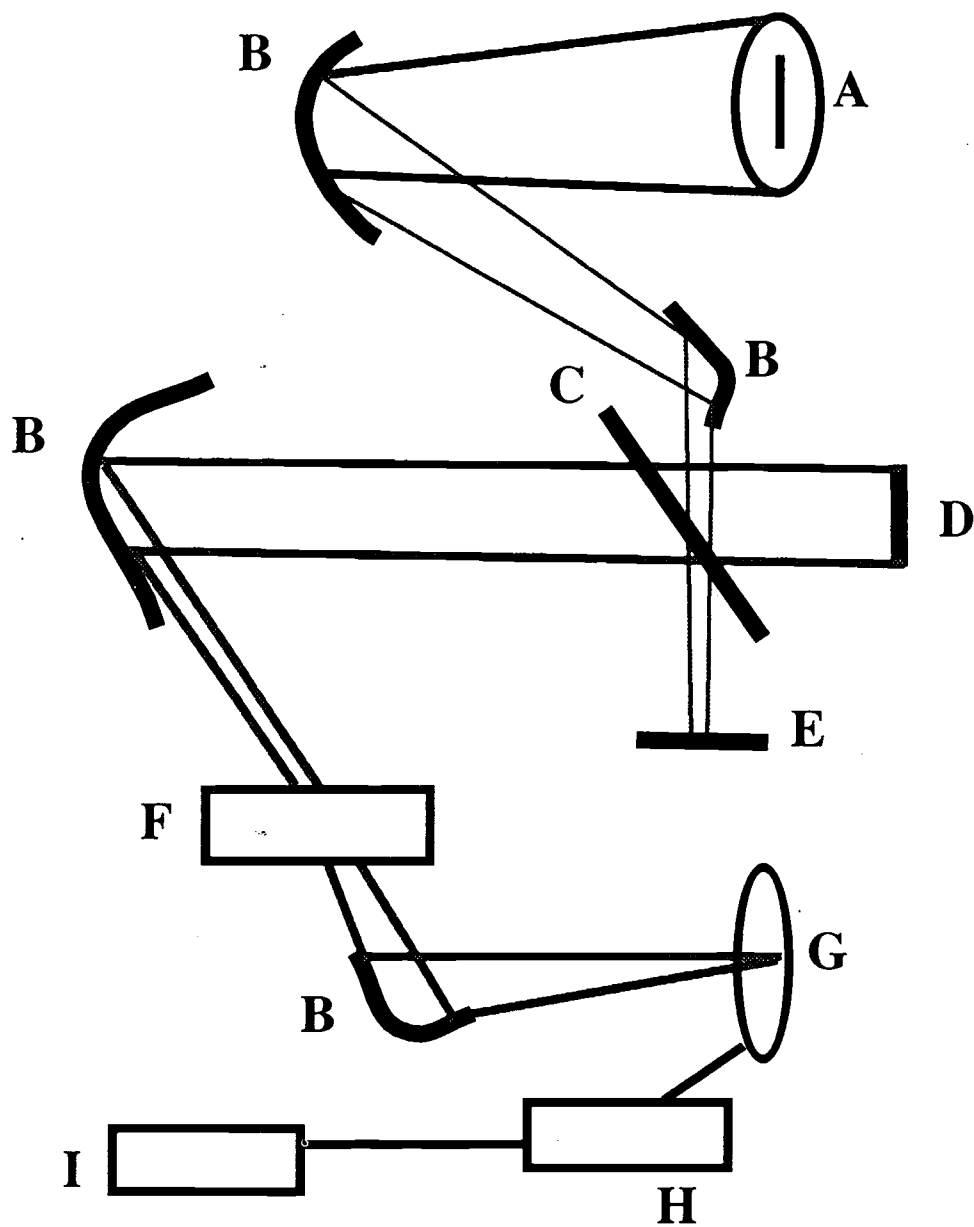


Figure 12-7

Schematic diagram of a Fourier transform infrared spectrometer (FTIR). A, source; B, parabolic mirrors (optics); C, beam splitter; D, fixed mirror; E, movable mirror; F, sample; G, detector; H, computer; I, output.

optical system is known as a *Michelson interferometer*. The modulated beam passes through the sample (Figure 12-7F) and into the detector (Figure 12-7G). The output from the detector (the interferogram) is analyzed by a computer (Figure 12-7H) using a technique known as *Fourier transform analysis* to obtain the spectrum (Figure 12-7I). Unlike traditional infrared spectrometers, the FTIR does not require a monochromator, and the entire spectrum is obtained in a very short time. Although the optics must be finely calibrated, the number of moving parts in the FTIR spectrometer is small.

FTIR yields spectra of higher quality than traditional IR spectrometry.

Fourier transform instruments measure the entire spectrum simultaneously rather than sequentially (this is the Fellgett advantage), which is particularly useful when measuring biological samples that may degrade in a short time. In addition, a larger aperture may be used (the Jacquinot advantage), allowing more infrared radiation to pass through the sample. Fourier transform instruments are capable of measuring very weak signals. The quality of the spectra obtained using FTIR is undoubtedly superior to that of spectra obtained using traditional instruments. Most instruments can be calibrated to give enlargements of very small portions of a sample, allowing the investigator to focus on one signal. Finally, it is much easier to maintain a constant temperature in the Fourier transform instruments, which can be an advantage when studying biological samples. Figure 12-8 is an example of a Fourier transform spectroscopy.

Applications of Infrared Spectrometry

Fourier transform is used to study:

- 1) *structure of proteins*
- 2) *base pairing in nucleic acids*
- 3) *behavior of lipids*
- 4) *binding of drugs to DNA*
- 5) *penetration of water into micelles*
- 6) *protein interaction*
- 7) *membrane structure and function.*

Among the chemical phenomena of biological interest that have been intensively studied using FTIR are the global structure of proteins (through analysis of the amide bands), base pairing in nucleic acids (through analysis of the carbonyl and N-H stretching bands), and behavior of lipids in natural membranes (through analysis of the C-H stretch). The binding of drugs to DNA, penetration of water into micelles, hydration of nucleic acids, protein interactions, and membrane structure and function have all been studied using FTIR.

With the advent of Fourier transform infrared spectrometers, it has become possible to obtain detailed information on a small range of wavenumbers. Information that remained hidden in traditional infrared spectra can be revealed using Fourier transform instruments. This technique offers great potential for the study of the structure, motions, and functions of proteins, enzymes, nucleic acids, and membranes.

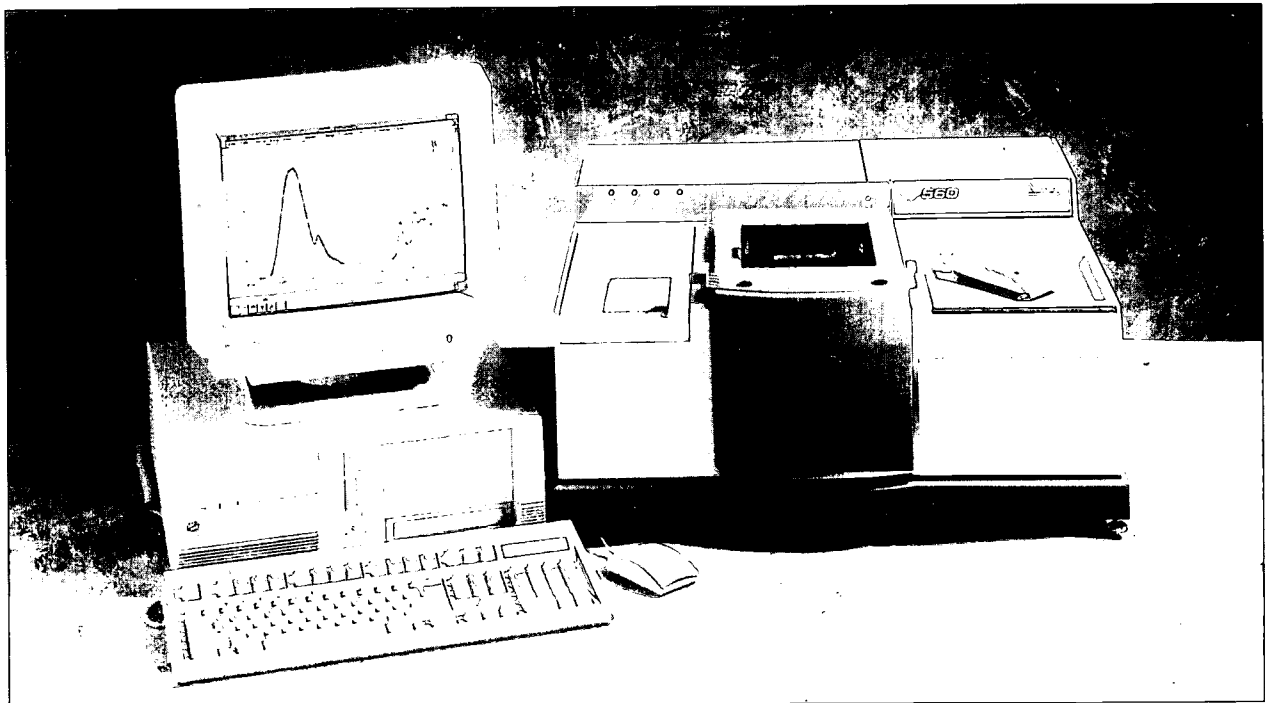


Figure 12-8
A Fourier transform infrared spectrometer
(*Courtesy Nicolet Instruments, Inc.*)

Questions Commonly Asked by Students

Question 1: How can I interpret the following infrared spectrum? (See Figure 12-9, page 218.)

There is no one best way to begin interpretation of an infrared spectrum. A good benchmark is the area around $3,000\text{ cm}^{-1}$. The C-H stretching signals in this area give information regarding presence or absence of carbon-carbon double bonds. If there is no signal immediately above $3,000\text{ cm}^{-1}$, there are no vinylic hydrogen atoms and probably no carbon-carbon double bonds. If there is no signal immediately below $3,000\text{ cm}^{-1}$, there are no hydrogen atoms attached to sp^3 -hybridized carbons, and probably no carbon-carbon single bonds. The spectrum shown has no signal immediately to the left of $3,000\text{ cm}^{-1}$; therefore, its structure probably has no carbon-carbon double bonds.

It is frequently useful to eliminate the presence of functional groups first. For example, if the carbonyl stretching signal is absent, the compound cannot be a carboxylic acid, ester, ketone, aldehyde, etc. Similarly, if the O-H stretch is absent, the compound is not an alcohol. Comparison of the unknown spectrum (see Figure 12-9) with Table 12-2 indicates that the compound is probably not an aldehyde, ketone, carboxylic acid, or any other compound containing a carbonyl group because the stretching signal near 1700 cm^{-1} is absent from the spectrum. However, the strong, broad band near $3,400\text{ cm}^{-1}$ is characteristic of alcohols.

*Use of the infrared spectrum alone may or may not be sufficient to positively identify a compound. In this example, the compound has been shown to be an alcohol whose structure contains no carbon-carbon double bonds. In combination with other spectroscopic techniques (nuclear magnetic resonance, mass spectroscopy, etc.) and various wet chemical tests, most compounds can be identified. The unknown compound in Figure 12-9 is *t*-amyl alcohol. It would be difficult to confirm this identity based on infrared spectroscopy alone, because many alcohols have similar spectra; however, a combination of the methods listed above would readily identify the compound.*

Question 2: How can I prepare samples for infrared spectroscopy?

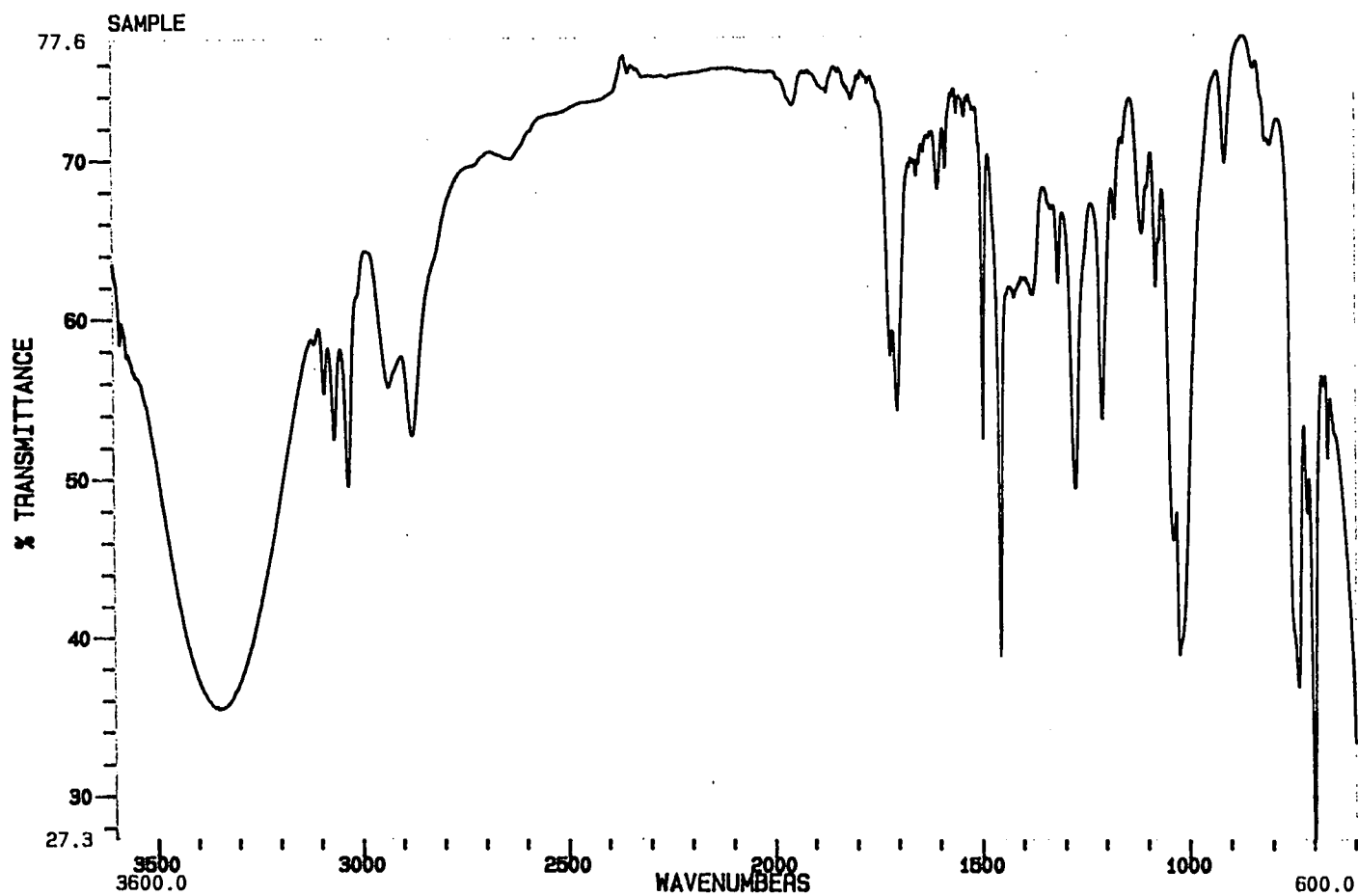
Most beginning students run liquid samples on the infrared spectrometer. The salt plates (usually sodium chloride) that are used to contain the sample should be clear and colorless, but are rapidly etched and turn cloudy on exposure to humid air. Salt plates must be stored in a container placed within a desiccator (commonly charged with anhydrous calcium chloride).

Always wear gloves when handling the salt plates. Inexpensive disposable polyethylene gloves are fine. Handle the plates by their edges only. Place one or two drops of your sample on one of the plates and cover with the other plate. If your sample is volatile, more sample may be required. Place the plates in the sample holder and secure. Because the salt plates are brittle and will crack under pressure, careful handling is necessary.

After obtaining your spectrum, be sure to clean the salt plates before storage. Some samples may be simply wiped off of the plates using a tissue. Others require gentle washing with a solvent. If you are directed to clean the salt plates with a solvent, wear gloves and always work under a chemical fume hood. Never wash the salt plates with water! Be sure to store the salt plates in the desiccator provided.

Question 3: What are some practical applications of infrared spectroscopy?

Many people first encounter infrared spectroscopy as a tool in the identification of organic and biomolecules. Infrared spectroscopy has been used in the identification and characterization of a wide range of commercial polymers and plastics, including many packaging materials, membranes, and plastic wraps used in the food industry. In addition, many natural membranes, such as the cell membrane, can be studied using infrared spectroscopy. Structures and motions of many complex molecules such as enzymes and nucleic acids can be studied. Knowledge of these structures and motions is frequently critical in the design and synthesis of new drugs and in medical treatment.



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Figure 12-9
Infrared spectrum of an unknown compound.



Gary Lyon (at left) demonstrates the use of Fourier transform infrared spectroscopy.

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Most organic chemistry textbooks contain one or more chapters on the theory of infrared spectroscopy and the interpretation of infrared spectra. These books can be excellent sources of information regarding specific stretching and bending signals. *The Spectrometric Identification of Organic Compounds* by Silverstein, Bassler, and Morrill (1991) contains an excellent chapter on infrared spectroscopy which is invaluable when interpreting spectra. The *CRC Handbook of Chemistry and Physics* (1994) contains "Infrared Correlation Charts" (sometimes called Colthup charts) that condense a great deal of information into a few pages. Finally, when interpreting an infrared spectrum, it is frequently useful to compare an unknown spectrum to various known spectra. An excellent compendium of published spectra is *The Aldrich Library of Infrared Spectra* (1981). Several sites that deal with spectroscopy are currently available on the Internet. Among these are the electronic edition of the newsletter of the Society for Applied Spectroscopy at <http://esther.asu.edu/sas/epstein/sas.html> and "Titles and Abstracts for Applied Spectroscopy" at <http://esther.la.asu.edu/sas/journal/asu49n8/asu49n8.html>.

About the Author

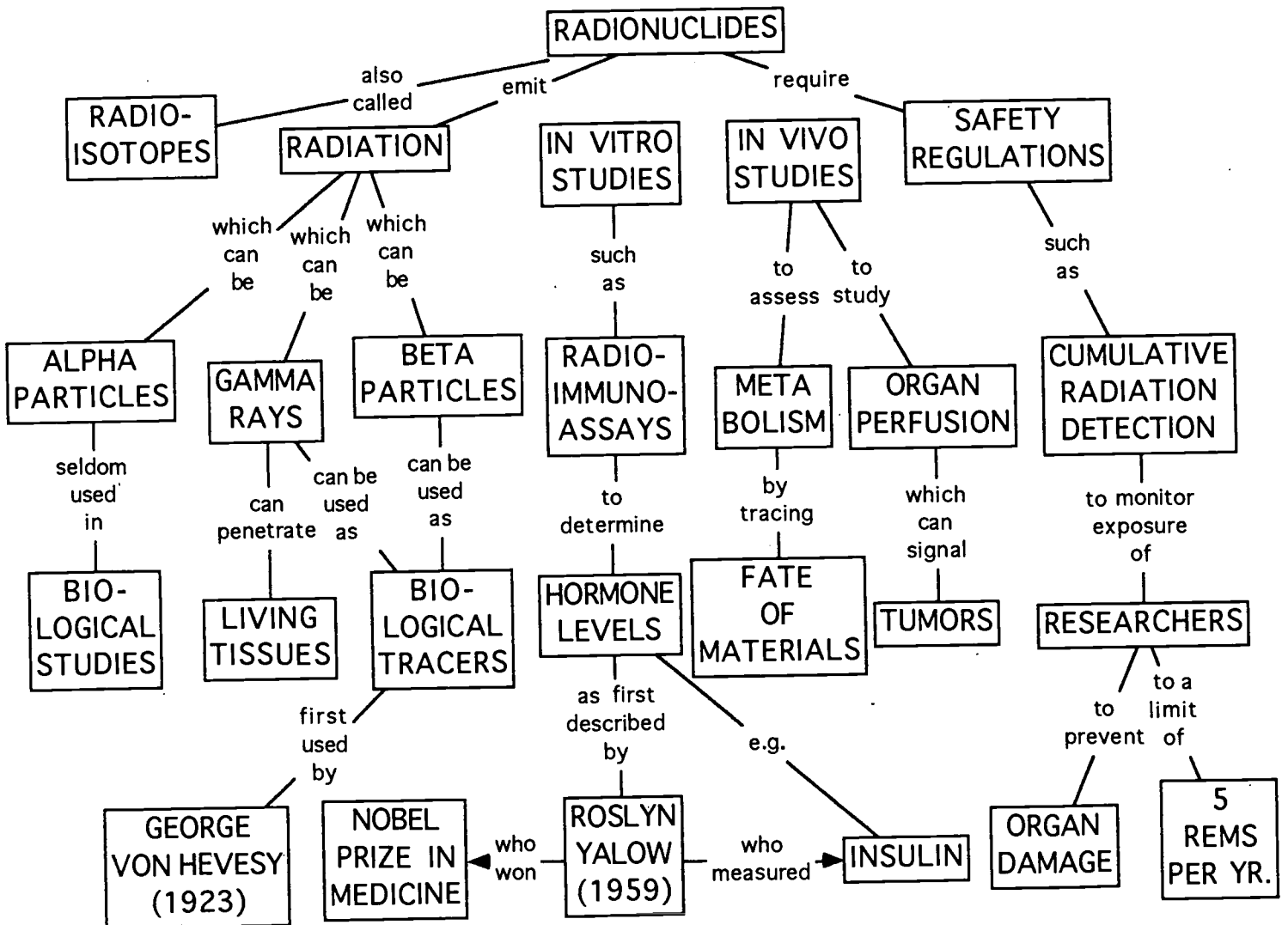
Gary L. Lyon received an MAT from Drake University in Des Moines, IA, and has taught inorganic, organic, and analytical chemistry at Drake University and Des Moines Area Community College in Boone, IA. He is currently employed at the Center for Scientific and Mathematical Literacy at Louisiana State University in Baton Rouge, LA, where he is also a science education doctoral student.

RADIONUCLIDES

13

Kenneth E. Griswold, Jr.
with commentary by *Daphne C. Jewell*

- Overview**
1. Characteristics of radionuclides
 2. Radiation safety
 3. Radiation detection
 4. Current and future uses of isotopic tracers



Radionuclides

A radionuclide is a species of an atom that exhibits radioactivity.

Throughout history, scientists have sought ways to study the metabolic fate of materials after they are introduced into organisms. In 1923, George von Hevesy accomplished this by placing a plant into a solution containing a radioactive form of lead and following the translocation of this isotope into the plant tissue. This was the first documented use of isotopic tracers in biology. Today, inexpensive radioisotopes of nearly all physiologically important elements are available for use as biological tracers. These radioisotopes can be incorporated into the chemical makeup of virtually all biomolecules to serve as a way of monitoring *in vivo* processes or to be used as reagents in *in vitro* analyses. The radioactivity emitted from these tracers is detected and measured by *autoradiography*, *dosimetry*, and *scintillation*. This chapter discusses the physiochemical nature of radionuclides and the characteristics of the methods that are commonly used to measure them.

Characteristics of Radionuclides

The term nuclide has replaced the term isotope.

The term *isotope* has traditionally been used to describe forms of all elements. This usage is not always correct because an isotope, by definition, is two or more forms of the same element that have the same atomic number, different atomic masses, and same chemical properties. It is more appropriate to use the term *nuclide* when referring to any particular atom. A nuclide is any atomic nucleus with its corresponding orbital electrons. The same definitions apply to the terms *radioisotope* and *radionuclide*, except these forms of an atom also emit radiation.

Orbiting electrons are held together by:

- 1) centrifugal force
- 2) binding energy

The nuclear constituents comprise the nucleon.

Negatively-charged electrons that encircle the nucleus of a stable atom are held in place by (1) centrifugal force and (2) binding energy, which is expended by the atom for no other effect than to maintain the electrons in their proper orbits. The positively charged protons and the neutrons that have no charge also expend energy that serves to bind the nucleus together in a stable configuration. The protons and neutrons of an atom are collectively referred to as the *nucleon*. The stability of the nucleon is maintained by a defined ratio of protons to neutron of any atom.

Energy is transferred from the nucleon, in the form of radiation if the proton-to-neutron ratio of an atom is altered. This energy loss causes the atomic mass of the atom to change. These events restore nuclear stability and change the radionu-

clide into a new nuclide which may or may not be stable. The process, called *radioactive decay*, continues until a stable element is formed.

At the beginning of the 20th century, Mme. Marie Curie designed an experiment in which she placed a piece of the radionuclide, *radium*, into a small hole that was drilled into a lead block. She also introduced a magnetic field perpendicular to the opening of the hole, placed a piece of unexposed photographic film above the hole, and encased the entire device to prevent exterior light from impinging on the film. She removed the film after an appropriate period of time and developed it. The film was darkened to the left of the hole (near the positive pole of the magnet), to the right of the hole (near the negative pole of the magnet), and immediately above the hole. She concluded that the radium had emitted three different types of radiation which had darkened the film. She named these emissions *alpha particles*, which were positively charged; *beta particles*, which were negatively charged; and *gamma particles*, which had no charge.

It later was shown that most radionuclides actually decay by alpha and/or beta emissions followed by the subsequent release of gamma radiation. Also, the decay schemes of radionuclides are not altered when they are combined with other chemicals or subjected to changes in pressure or temperature.

An alpha particle consists of two protons and two neutrons. These particles have an energy ranging from 3-9 million electron volts (MeV) when they are ejected from the nucleus of a radionuclide. They can attract electrons from most materials they pass through, causing the target material to ionize. They will also transfer some of their energy to the nuclei of the atoms of the target material. These interactions result in alpha particles losing their energy. The energy loss is so rapid that even a single sheet of notebook paper can absorb most alpha particles. Alpha radiation is seldom measured in biological studies because it lacks penetrability and because most alpha-emitting radionuclides are naturally occurring heavy metals that are not normally found in biological systems.

A beta particle is actually an electron that originates in and is ejected from the nucleus of some radionuclides. There are two types of beta particles: (1) positively-charged ones called *positrons* and (2) negatively charged ones called *negatrons*. A positron is produced when a proton is converted to a neutron

Alpha particles are positively charged.

Beta particles are negatively charged.

Gamma particles are neutral.

Radionuclides decay by alpha and/or beta emissions followed by release of gamma radiation.

Alpha particles have limited use in biological studies.

Beta particles arise from the nucleus of some radionuclides.

Two types of beta particles exist:

- 1) positively charged — positrons*
- 2) negatively charged — negatrons*

to a neutron in a neutron-deficient radionuclide. A negatron will be emitted from a proton-deficient radionuclide, as a neutron is converted to a proton. Both these processes do not change the mass number of the radionuclide, but the atomic number *decreases* with positron emissions, and the atomic number *increases* with negatron emission.

Beta particles lose energy by exciting atoms they come into contact with. This lost energy is in the form of x-radiation.

The term E_{max} is equivalent to the total energy available from nuclear decay and is characteristic of each nucleotide.

Beta radiation is more penetrating than alpha radiation.

Examples of beta-emitting radionuclides are:

- 1) carbon-14
- 2) tritium.

The amount of voltage produced by a particular gamma ray is unique to the emitting radionuclide, allowing its identification.

Gamma rays lose their energy by:

- 1) photoelectric effects
- 2) Compton effect
- 3) pair production.

Beta radiation has an energy of 0-3 MeV. Each beta particle has a unique E_{MAX} that can be used to identify the radionuclide that emits the particle. Some radionuclides are pure beta emitters, while others emit both beta particles and gamma radiation. Unlike alpha radiation, beta particles lose energy when they repel electrons to form ion pairs in atoms of material that they penetrate. Ultimately this energy is lost from these atoms in the form of x-radiation. Beta particles can also lose energy by exciting the nuclei of the atoms of materials they come in contact with. Positrons can also collide with electrons in material which effectively destroys both. This process is called *annihilation*. The rate of energy loss from beta radiation is slower than that of alpha particles. This increases their penetrability to approximately 1,000 times greater than the penetrability of alpha particles. But, an inch-thick piece of wood can act as an effective shield against beta radiation, so the rate of energy loss is still considerably rapid. There are several biologically significant beta-emitting radionuclides, such as carbon-14 and tritium. Therefore, many applications have been designed for their use in biology and medicine.

The emission of alpha particles, negatrons, and positrons energizes the nucleus of the parent radionuclide. This energy is immediately released as gamma radiation. The amount of voltage produced by a particular gamma ray is unique to the emitting radionuclide and can be used to identify that radionuclide. Since gamma rays are pure energy emissions they have no mass or charge. Generally, gamma rays lose their energy in three ways. First, low-energy gamma rays (< 0.5 MeV) transfer their energy to the orbital electrons of matter they strike in a process called the *photoelectric effect*. This causes these electrons to be ejected from their orbit or to form ion pairs. The ejected electrons transfer this excess energy to other electrons in their path. Gamma rays of intermediate energy (0.5-1.0 MeV) cause an additional event to occur when they strike matter. This second event is called the *Compton effect*. Because only a portion of the energy of these rays is required to produce the photoelectric effect, the

Penetration of gamma rays depends upon:

- 1) strength of the ray
- 2) amount of the radionuclide
- 3) material it is exposed to

Examples of gamma-emitting radionuclides are radioisotopes of iodine, iron, and phosphorus.

Units associated with radioactivity are:

- 1) Curie
- 2) Becquerel
- 3) dps

Specific activity is the amount of radioactivity per unit weight in a sample.

remaining energy is ejected from the atom in the form of a second gamma ray. This second gamma ray is called *Compton effect* or *Compton Scatter*. A third event, called *Pair Production*, occurs when high energy gamma rays (> 1.02 MeV) penetrate atoms of target material to near their nuclei. This penetration causes beta particles and positrons to be produced. The beta particles are ejected from the atom. They produce the effects previously described as they interact with atoms in the target material. The positron collides with an orbital electron in the atom from which it originated and both particles are annihilated. This annihilation produces two gamma rays that are ejected from the atom.

Gamma rays can penetrate to considerable distances into or through the materials they strike. The extent of the interactions they produce in the material is a function of the strength of the rays and the amount of gamma-emitting radionuclide to which the matter is exposed. There are many radionuclides that emit gamma rays, such as radioisotopes of iodine, iron, and phosphorus. This type of radiation is also easy to detect, so these are often used as tracers in biology, medicine, and industry. Gamma radiation is more dangerous to work with than alpha and beta radiation, so lead or concrete barriers are often used to shield anyone who is working with gamma-emitting materials.

Neutron-deficient radionuclides can also decay by a process called *electron capture*. The excessive protons in this type of radionuclide "capture" electrons from the electron cloud surrounding the nuclide and convert them to neutrons. The orbital electrons then rearrange, releasing energy in the form of X-radiation.

All radionuclides decay at a constant rate to a stable form and this rate is expressed as the *half-life*. Half-life is the amount of time necessary for a certain radionuclide to lose half its energy. The half-life for a specific radionuclide is unique to that element and is a very important value to know when selecting the appropriate radionuclide to use in tracer experiments, as well as when disposing of radioactive waste. *Disintegrations per second* (dps) is a term used to express the rate of radioactivity in a sample. This value is currently expressed in *Curies* (Ci). The proper definition of a Curie is the activity of a sample decaying at a rate of 3.7×10^{10} dps. The Becquerel (Bq) is the SI unit used to express radioactivity and is the one that will be used in the future to express radioactivity. $1 \text{ Bq} = 1 \text{ dps}$; therefore, $1 \text{ Bq} = 2.703 \times 10^{-11} \text{ Ci}$ (or $3.17 \times 10^{-11} \text{ Bq/Ci}$). *Specific activity* is

the most significant unit to know for those working with radioisotopes in biology. It is the term used to express the amount of radioactivity per unit weight in a sample and is expressed in Curies/gram.

Most nucleotides are produced by:

- 1) *fission reactions*
- 2) *neutron activation*
- 3) *generators.*

There are approximately 280 non-radioactive nuclides, and over 900 radioactive ones. Most nuclides are artificially produced by fission reactions, neutron activation, or in generators. *Fission reactions* take place in heavy radionuclides. The original radionuclide is converted to smaller ones, causing the release of gamma radiation and neutrons. This process is controlled in a nuclear reactor. *Neutron activation* involves the capture of a neutron into a stable nucleon, resulting in the emission of a gamma ray and the production of radioactive form of the parent nuclide. The radionuclide by-products of fission and neutron activation are harvested, and then each separate radionuclide can be isolated and marketed. Radionuclides are available from a variety of suppliers, but a special license is required to purchase them. *Generators* can be used to bind a "parent" radionuclide that has a long half-life to ion-exchange resin in a chromatographic column. The "parent" decays to desired "daughter" radionuclides that are eluted from the column by conventional chemical means when they are needed for use as tracers. Generators are most often used in nuclear medicine.

Radiation Safety

Radiation protection is required for all personnel who use radioactive material. Title 10 of the Code of Federal Regulations details the federal requirements for radiation protection and safety. The Nuclear Regulatory Commission controls the receipt, storage, use, and disposal of radioactive materials; the Food and Drug Administration controls the use of these materials in the medical setting; and the Department of Transportation controls the shipment of radionuclides. Most states also have additional rules for their use. Central to the regulations of all these agencies is that all those who work with radionuclides have documented training on the proper use and handling of radioactive materials and must be knowledgeable of radiation safety.

The most practical factors to minimize any damaging effects to personnel are: (a) distance, (b) shielding, and (c) duration of exposure. As persons move away from a radiation source they will receive less radiation. One meter or more is a reasonable working distance from any source of radiation. This ex

A distance of one meter or more away from the typical source of radiation is considered safe.

Shielding is required when working with beta and gamma rays.

Personnel working with radionuclides are routinely monitored for evidence of exposure to radiation.

plains why personnel use long-handled tongs and remote handling devices when manipulating large quantities of radionuclides. Shielding is necessary when working with beta and gamma emitting radionuclides. Shields must be of sufficient density and thickness to reduce exposure to acceptable limits. The amount of time an individual is exposed to radiation also increases total cumulative exposure. Therefore, the total amount of time personnel spend working in the presence of radionuclides is also carefully monitored.

Other general requirements for using radionuclides are: (1) records must be kept of the receipt, transfer, and disposal of radioactive material; (2) all radioactive sources must be labeled and stored in a way that will prevent personnel from undue exposure to them; (3) disposable gloves and protective clothing must be worn when working with a radionuclide; (4) radioactive materials can only be used in specially designed and designated areas; (5) shielding and remote handling devices may be required, depending on the specific activity of the radionuclide; (6) mouth pipetting is not allowed in the work area; (7) eating, drinking, and the application of makeup is not allowed in the work area; and (8) radioactive waste must be stored in marked containers, disposed of by proper agencies, and not allowed to enter normal routes of trash or sewage disposal system. Additional requirements may be specified by regulatory agencies in your area.

It should be noted that 0.5 rems per year is the maximum exposure for the general public, 5 rems per year for those working with radiation.

Since it is impossible and impractical to totally shield personnel who work with radionuclides from all of the ionizing effects of these materials, the amount of radioactivity to which these persons are exposed must be routinely monitored. Personnel and area-monitoring devices designed for this purpose are described in the next section. The value which is the most significant in assessing personnel exposure is the roentgen equivalent (rem). The rem is the dose of ionizing radiation that can cause an amount of injury to human tissue equivalent to 100 ergs of absorbed energy per gram of absorber. One hundred ergs of absorbed energy per gram of absorber is also known as radiation absorbed dose or *rad*. The maximum level of exposure to radiation is 0.5 rems/year for members of the general public and 5 rems/year for radiation workers. Another unit often used in the field of radiation safety is the *roentgen* (R). A roentgen is the amount of gamma or X-radiation that can produce one electrostatic unit in 0.001293 g of air. Organ damage

Tissue damage from nuclear radiation is cumulative.

from radioactivity is cumulative. Any persons who are exposed to excessive rems of radiation must be quickly decontaminated, removed from any further exposure, and have their health status monitored to detect any possible organ damage.

Radiation Detection

Devices used to detect and measure nuclear radiation include:

- 1) *photographic emulsions*
- 2) *ionization chambers*
- 3) *scintillation detectors.*

Devices that are used to detect and measure nuclear radiation are designed to measure the ionization or excitation of material with which the radiation interacts, rather than to measure the radiation directly. These devices are *photographic emulsions*, *ionization chambers*, and *scintillation detectors*.

Photographic Emulsions

The photographic emulsion can be used to detect any type of ionizing radiation and is the oldest and most widely-used method to detect radioactivity. Becquerel discovered radioactivity in 1896 by placing a piece of potassium uranyl sulfate on a tightly wrapped, unexposed photographic plate. When he developed the plate, he found an exposed area on the plate that was exactly the same shape as the piece of uranium salt. He concluded that the salt produced some type of ray that penetrated the cover of the plate and exposed the film as light would do.

Photographic film is used to monitor exposure to radiation in badges worn by personnel working with ionizing radiation.

Today, photographic film is used in the construction of the film badges that must be worn as monitoring devices by personnel who work with ionization radiation. The badges can be worn as a pin or ring. The badge consists of a piece of unexposed photographic film that is placed into a light-proof holder and covered by different filters. A cadmium filter is placed over one third of the film; one third of the film is covered by a copper filter; and the final one third is covered by both a cadmium and copper filter. X-radiation can activate the portion of the film that is covered by the copper filter alone. Beta radiation can penetrate the portions covered by either the copper or cadmium filters, but not the copper and cadmium combination. Gamma radiation can penetrate *all* filters. Periodically, the film is removed from the badges, developed, and the amount of film that is exposed is measured.

These measurements provide a record of the types and amounts of radiation a person has been exposed to since the last film change. Another technique that uses photographic film to detect radiation is *autoradiography*. A radionuclide or a ra-

dionuclide-labeled compound is first assimilated into a specimen for use as a tracer. The specimen can be a biomolecule, cellular organelle, cell, or intact organism. The labeled specimen is then wrapped along with a piece of undeveloped film. When the film is removed and developed, the distribution of the radionuclide in the material can be determined. Conventional black-and-white or X-ray film and developer are often used in this technique. These are inexpensive and available in camera supply stores.

Ionization Detectors

Ionization detectors are sealed containers of argon, helium, hydrogen, neon, or air into which a positive and negative electrode have been inserted. The electrodes are connected to an ammeter and a power supply. The ammeter is used to detect any current flow in this circuit, and the power supply is used to create a voltage differential between the two electrodes. Ionizing radiation that strikes the detector produces ion pairs in the gas molecules, with the positively-charged member of the pair migrating to negative electrode and the negatively-charged member migrating to the positive electrode. These primary ions create a current flow that is directly proportional to the amount of radioactivity striking the detector. If the voltage in this circuit is increased, the primary ions can be energized to a point at which they cause further ionization of other gas molecules. This increased ionization improves the sensitivity of these detectors to alpha, beta, gamma, and X-radiation. Devices that fall into this category of instruments include *Geiger-Muller counters*, *pocket dosimeters*, *proportional counters*, and *ionization chambers*. Geiger-Muller counters, proportional counters, and ionization chambers are used as work area monitoring devices, and pocket dosimeters are used for personnel monitoring.

To monitor for alpha, beta, gamma, and x-radiation, instruments such as Geiger-Muller counters, pocket dosimeters, proportional counters, and ionization chambers are used.

Scintillation Detectors

An energized electron and/or gamma ray that is emitted from a radionuclide can impart its energy to the orbital electrons of many target materials. The excited electrons in the target material subsequently lose this energy as photons of light. Target materials that are capable of this response are called *fluors* or *scintillators*. The number and strength of the photons of light emitted by a fluor are proportional to the number and strength of the radiation that produces them. Scintillation detectors are designed to detect and measure the amount of light emitted by a fluor when a radioactive sample is placed in contact with it.

Material capable of emitting photons of light are called fluors or scintillators.

The number and strength of photons emitted by fluors are proportional to the number and strength of the radiation producing them.

Figure 13-1 is a block diagram of the type of scintillation counter that is most often used to measure gamma radiation. The fluor in this counter is a crystal of sodium iodide, fortified with 1% thallium. A well must be milled into the crystal to hold containers of the gamma-emitting sample while they are being counted. A photomultiplier tube is placed in direct contact with the crystal, and the crystal and tube are encased in metal to prevent them from coming in contact with moisture and exterior light. Photomultiplier tubes convert the photons of light given off by the fluor into pulses of electrical current. The number of the electrical pulses produced in a tube is proportional to the number of radioactive emissions interacting with the fluor. A specific amount of voltage is produced in a tube by this process that is called the *pulse height or photopeak*. A particular photopeak is directly proportional to energy of the incident gamma ray that is produced by the radionuclide being measured. Each gamma-emitting radionuclide produced gamma rays that have a unique photopeak. A power supply is connected to the photomultiplier to produce the proper voltage needed to operate the tube. Photomultipliers also amplify the electrical pulses and send them to a preamplifier. The preamplifier reduces distortion in the electrical pulses.

The photomultiplier tube converts photons from fluors into electrical current, and the resulting amount of voltage produced is referred to as pulse height or photopeak.

Each gamma-emitting radionuclide produces gamma rays that have a unique photopeak.

The pulses then move to an amplifier that further increases the amplitude of these pulses. The voltage of the amplified pulses is finally measured by the pulse-height analyzer, and only those pulses within the photopeak are allowed to continue through the circuit. Devices called *discriminators*, are used to set a *voltage window* around the photopeak of the radionuclide being measured. Some counters have windows that are pre-set for specific radionuclides by the manufacturer, while others have discriminators that the operator must use to set the appropriate energy window. Only the pulses of energy that fall within the window are counted by a scaler that is connected to the pulse-height analyzer. Scalers can be set to accept a pre-set number of counts, and the data are expressed as total counts. Or the pulses can be counted for a pre-set amount of time and the data expressed as counts per minute. Usually, the scaler is interfaced with a computer that can analyze these data. Robots can be used to index a series of samples into and out of the well. Multiple pulse-height analyzers and scalers can also be incorporated into a single instrument that will enable the simultaneous detection and counting of emissions from multiple gamma-emitting isotopes that may be contained within a single sample. Instruments used in nuclear medicine for organ imaging and in industry to measure gamma radiation being emitted over a large

A scaler is an electronic counter.

The computer produces a two- or three-dimensional image of the structure giving off the gamma rays.

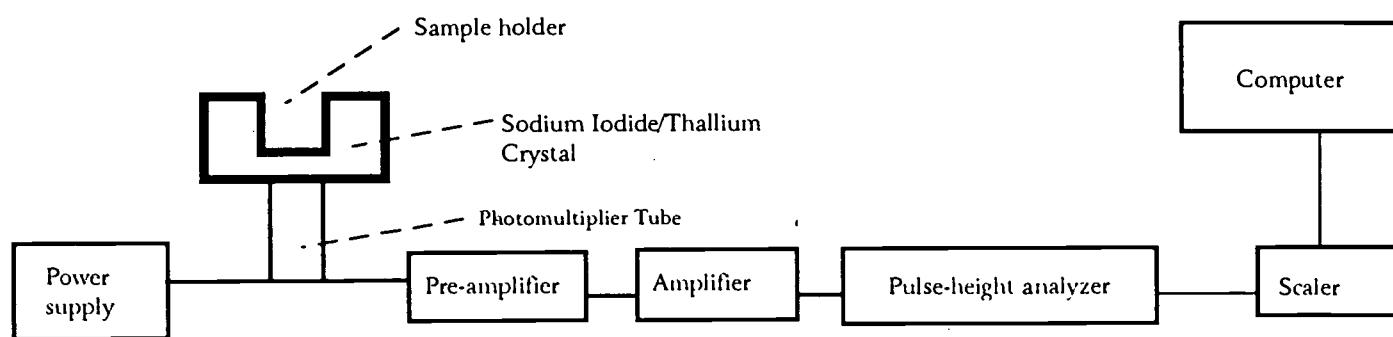


Figure 13-1
Block diagram of a solid crystal scintillation counter used for gamma radiation counting.

area usually use an array of crystals and/or phototubes to simultaneously scan an area. The phototube assembly in this array is connected to a computer that is used to construct a two- or three-dimensional image of the structures emitting the gamma radiation. Figure 13-2 shows a simple liquid scintillation counter that is used for beta radiation measurements. Beta particles cannot penetrate the walls of sample containers, so beta-emitting materials must be mixed with a combination of a solvent and a fluor in a vial prior to counting. Toluene, xylene, or pseudocumene are commonly used solvents, and complex organic compounds such as 2,5-diphenyloxazole (DPO) or 1,4-bis-2 (5-phenyloxazolyl)-benzene (POPOP) are used as a fluor. The beta particle first interacts with and excites the solvent molecules. The energy of the excited solvent molecules is then transferred to the scintillator, which gives off the energy in the form of light. The combination of the sample, solvent, and fluor is called a *scintillation cocktail*.

The sample, solvent, and fluor make up the scintillation cocktail.

Vials containing the cocktail are placed into the sample compartment of the beta counter for counting. Two photomultiplier tubes are located adjacent to the sample compartment, and the tubes are connected together in a coincidence circuit. Both tubes will simultaneously detect photons of light produced by the interaction of a beta particle with components of the cocktail, and the signal from the tubes is allowed to pass to the pulse-height analyzer. Any signals that arise independently in either tube are rejected by the coincidence circuit. The pulse-height analyzer and the remaining components of a liquid scintillation counter are the same as found in a solid crystal counter.

Current and Future Uses of Isotopic Tracers

In Vivo Studies

The most significant historical use of isotopic tracers has been to study the metabolism of substances in living systems. These systems utilize radiolabeled compounds exactly as they would non-labeled counterparts. This allows scientists to trace the metabolic fate of virtually all biologically-significant materials in isolated tissue, cell-free extracts, or intact organisms. Autoradiography and scintillation cameras are used to follow the spatial distribution of a labeled metabolite in an organism, while conventional scintillation counters are employed to detect and quantitate metabolites in materials isolated from the treated organism. Thousands of scientific papers that describe the dynamics of metabolism and the ultimate fate of virtually all com-

A metabolite is any product of intermediary metabolism.

Autoradiography and scintillation cameras are used to identify movement of the nuclide tracer.

Biologic tracers aid in the identification of tumors and alterations in tissue perfusion.

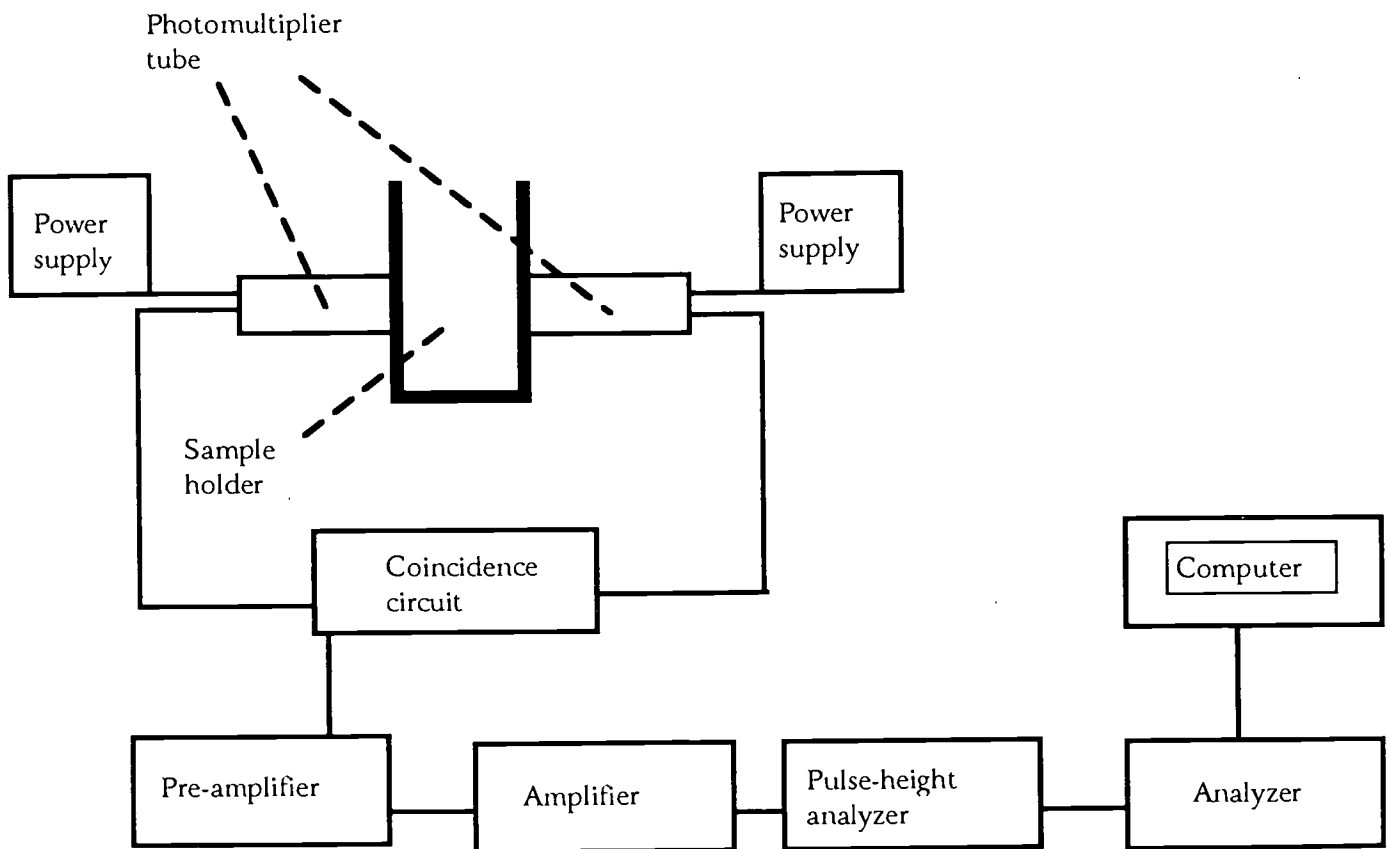


Figure 13-2
Block diagram of a liquid scintillation counter used for beta radiation counting.

Perfusion is the pumping of fluid through a tissue or organ via an artery.

pounds found in living systems are based on these techniques. They continue to be used routinely in the basic sciences and have also led to the development a whole field of medicine called *nuclear medicine*.

In vivo studies, designed to localize a tumor in an organ or to assess organ function, are routinely performed in nuclear medicine. In *in vivo* studies, a compound that will selectively concentrate in a organ is synthesized to include a radioactive tag with a short half-life. The compound is then administered, allowed to equilibrate in the structure of interest, and scanned with a scintillation camera. The distribution of labeled compounds can be followed by the camera as they are distributed in the body. Data obtained from these scans are interpreted by the nuclear medicine physician to confirm or refute a provisional diagnosis of tumor or organ perfusion dysfunction. There are hundreds of these techniques that are used today in a modern nuclear medicine department. These studies negate the need for expensive and dangerous exploratory surgery to confirm a diagnosis and are of little (or no) discomfort or danger to the patient.

***In Vitro* Studies**

Radioimmunoassay allows for the measurement of hormones, neurotransmitters, vitamins, drugs, and other substances found in tissues.

In vitro studies, called *radioimmunoassays* (RIA), are also conducted in nuclear medicine. Radioimmunoassay combines the ability to detect minute amounts of radioactively-tagged antigen with the specificity afforded by the reaction of an antigen with an antibody. This combination allows the quantitative measurement of hormones, neurotransmitters, vitamins, drugs, and other substances that often occur in nano- or femtomole levels of concentration in an organism. The technique itself was first described by Rosyln Yalow and Soloman Berson in 1959, who used it to measure the hormone, insulin. Dr. Yalow won the Nobel Prize in Medicine for her work with RIA, and she continues to be active in this field at this writing.

The basic analytical steps in an RIA are:

- (1) Isolate or synthesize the antigenic compound to be measured.
- (2) Inject a portion of the compound into a test animal to produce antibodies that will specifically react with the compound. Isolate and titer the antibodies for future use as reagents in the RIA.
- (3) Radiolabel and determine the concentration of another portion of the antigenic compound to be used as a reagent in the RIA.

Most radionuclides used in RIA are gamma emitters.

- (4) Use another portion of the compound to prepare standards and controls to be used in the RIA.
- (5) Label a series of tubes *standard, control, or unknown*.
- (6) Place equal amounts of the antibody and equal amounts of radiolabeled antigen in all tubes.
- (7) Pipette a predetermined volume of standard into the appropriately-labeled tube. (The concentration of each standard is known. Most RIAs require the preparation of a series of standards, rather than only one).
- (8) The same volume of a control sample is added to the *control tube*. (Control specimens is used to assess the accuracy and precision of the analytical method. The composition of standards and controls are essentially the same, except standard materials are dissolved in deionized water, and the radionuclides used for controls are incorporated into the same chemical matrix as the unknown.)
- (9) The same volume of the unknown specimen is added to the *unknown tube*.
- (10) Equal amounts of a buffer solution are added to all tubes, and the tubes are incubated for a predetermined amount of time to allow the antigen to bind to the active sites on the antibody. (The labeled and unlabeled antigen in each tube compete for binding sites on the antibody. If the concentration of the labeled antigen is greater than that of the unlabeled antigen, a greater proportion of the antigenic sites will be occupied by labeled antigen. The reverse will be true if the amount of unlabeled antigen in the tube exceeds that of its labeled counterpart. All unbound antigen will remain free in the buffer).
- (11) Separate the antigen-antibody complex from unbound antigen remaining in each tube.
- (12) Activate a scintillation counter and set the energy window for the radionuclide that was used as a tracer in the RIA. (Most radionuclides used in RIA are gamma-emitting isotopes and can be counted without any additional preparation. If the tracer is a beta-emitting isotope, each sample must be mixed in a *scintillation cocktail* before counting).
- (13) All containers are then sequentially placed into the sample well of the instrument and counted.
- (14) The known concentration versus counts obtained for each standard are used to construct a standard curve.
- (15) The concentrations of each control and unknown specimen are then determined by using the standard curve. Computer-assisted technology, robots, and mechanical pipettors can be used to automate these assays.

Radiolabeled gene probes are used to detect complementary DNA — to locate specific genes.

Another interesting in vitro application is in the field of molecular biology. Scientists have long been able to clone genes by a variety of methods, but were faced with the problem of locating a specific gene among the millions of genes that may occur in a genome. This problem has been solved by hybridizing the gene of interest with a radiolabeled gene probe. Gene probes can search and detect complementary sequences of DNA in the presence of large amounts of non-complementary DNA. The following is an example of how a DNA probe can be synthesized and used to locate a particular gene.

Insulin mRNA is used in producing reverse transcriptase, a DNA-RNA hybrid.

Pancreatic cells contain large amounts of insulin mRNA. This mRNA can be easily isolated and purified. If purified insulin mRNA is incubated with radiolabeled nucleic acids and the enzyme, *reverse transcriptase*, a DNA-RNA hybrid is produced. The DNA strand of the hybrid is synthesized from the labeled nucleic acids. In an additional step, the mRNA is replaced in a step-wise fashion by a second strand of DNA, and an intact DNA molecule is synthesized using the enzyme DNA polymerase. The newly-synthesized DNA, called *complementary DNA* (cDNA), can then be cloned from radiolabeled nucleic acids to produce a sufficient amount of the material to be used as an effective probe.

Southern Blotting is used to isolate specific genes and is important to molecular biology.

To locate the insulin gene in the genome of a human cell, the total DNA of these cells must be fragmented by restriction enzymes. Insulin cDNA is added to these DNA fragments and the mixture is heated. Heating causes the strands of all of the DNA helices to separate. When the mixture is cooled, DNA double helices re-form. The complementary strands in the re-formed DNA can come from the original molecules, or one strand can come from the gene fragment of interest and the other strand from the cDNA probe. The re-formed DNA hybrids are separated from other gene fragments by gel electrophoresis and the separated fragments are transferred to nitrocellulose sheets by a process called *blotting*. The location of the insulin gene hybrid on the blot can now be determined by autoradiography. This technique is known as *Southern Blotting*. Southern Blotting has been used to isolate thousands of biologically and medically significant genes, and it is one of the most important methods used in molecular biology today.

This chapter presents only highlights of the characteristics of radionuclide tracers, methods to detect and measure them, and some revolutionary ways we use them in biology and medicine. No attempt has been made to describe and explain any specific analytical methods, or to provide a bibliographic listing of isotopic tracer research. To do so would go far beyond the

scope of this chapter. Several excellent protocols are given in the references listed for those who wish to conduct such studies. As a note of caution, the damage caused by the ionizing effects of any radioactive material makes working with these chemicals a potential threat to both the analyst and the environment. No experiments should be attempted without formal training on the safe handling of these materials.

Questions Commonly Asked by Students

Question 1: Why is it dangerous to work with radioactive material?

Organ damage from radioactivity is cumulative.

Question 2: What characteristics of radionuclides make them useful as biological tracers?

Their penetrability is the key to their utility as biological tracers. Also, most radionuclides are easy to detect or image.

Question 3: What are the major differences in alpha, beta, and gamma radiation?

Alpha Radiation

1. *Their energy level is 3-9 MeV.*
2. *Energy is lost by ionizing the matter through which they pass or with which they collide.*
3. *Their energy loss rate is faster than that of beta rays, thus making alpha rays' penetrability less.*
4. *They are moderately dangerous.*

Beta Radiation

1. *Their energy level is 0-3 MeV.*
2. *Their energy is lost by ionizing the matter through which they pass.*
3. *Their energy loss rate is slower than that of alpha particles, thus increasing the penetrability of beta rays.*
4. *They are moderately dangerous.*

Gamma Radiation

1. *Their energy level is 0.5-1.02 MeV.*
2. *Their energy is lost by ionizing the matter through which they pass.*
3. *They have an unknown energy loss rate.*
4. *Gamma rays are more penetrating than alpha or beta particles.*
5. *Gamma rays are extremely dangerous.*

Question 4: What are the major differences between beta and gamma scintillation counters?

Beta Scintillation

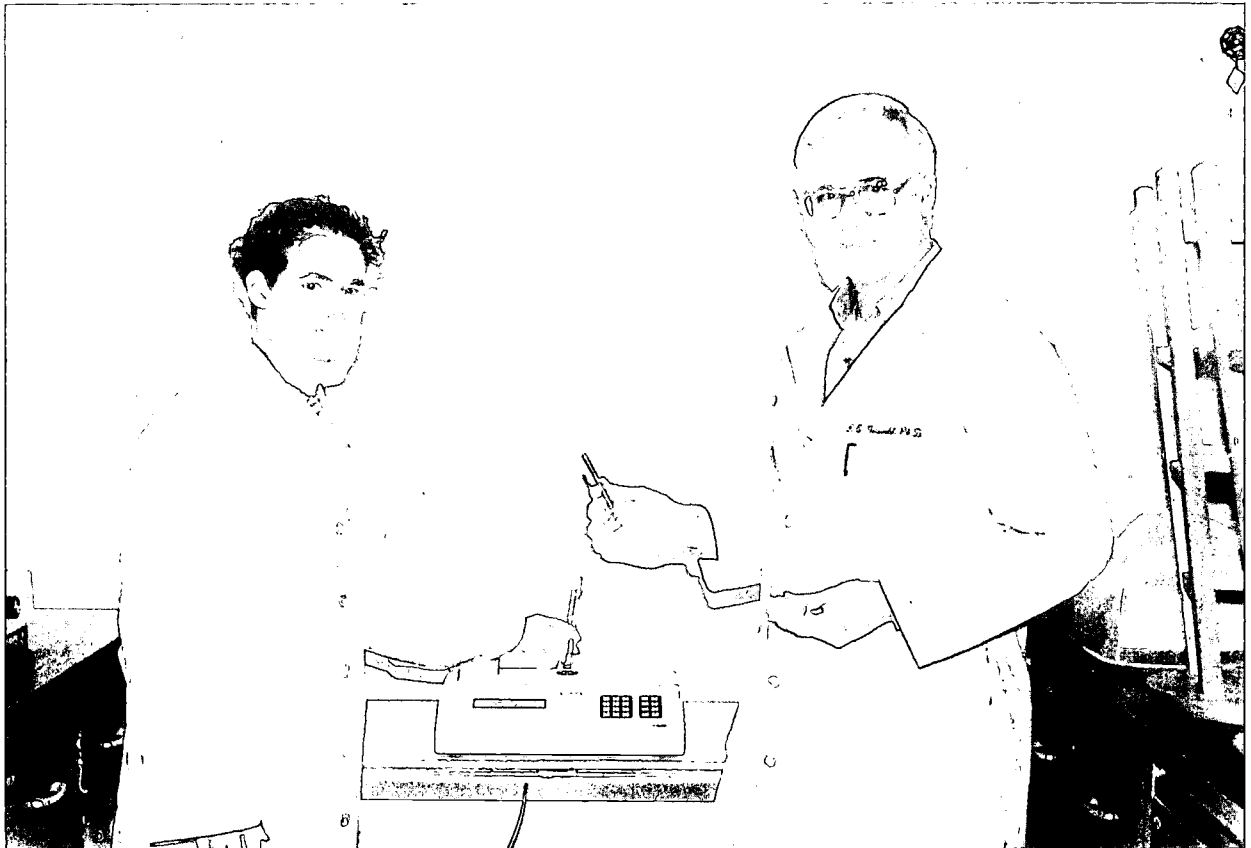
1. *Liquid material used as fluor.*
2. *Requires two photomultiplier tubes capable of reading a continuous energy spectrum.*
3. *Uses a wide window for measuring the spectrum of energies from the beta radiation.*
4. *Sample containers cannot be penetrated by beta particles; thus a scintillation cocktail is required.*

Gamma Scintillation

1. *Solid crystalline material is used as a fluor.*
2. *Requires a single photomultiplier tube.*
3. *Capable of reading a photopeak.*
4. *Uses a narrow window set for gamma radiation measurement.*
5. *Sample containers can be penetrated by gamma rays; thus no scintillation cocktail is required.*

Question 5: What other types of tracers are used in biology and medicine?

Fluorescent material and enzymes can also be used to label biologically -important compounds. Their use is limited to in vitro measurement and they lack the sensitivity of nucleotides.



Dr. Griswold stands behind an Isocomp Solid Crystal Gamma Scintillation Counter, holding a film badge in his left hand and a pocket dosimeter in his right hand. Heath Hanna, a senior medical technology major at Louisiana Tech University, is preparing to use the scintillation counter.

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Commentary by

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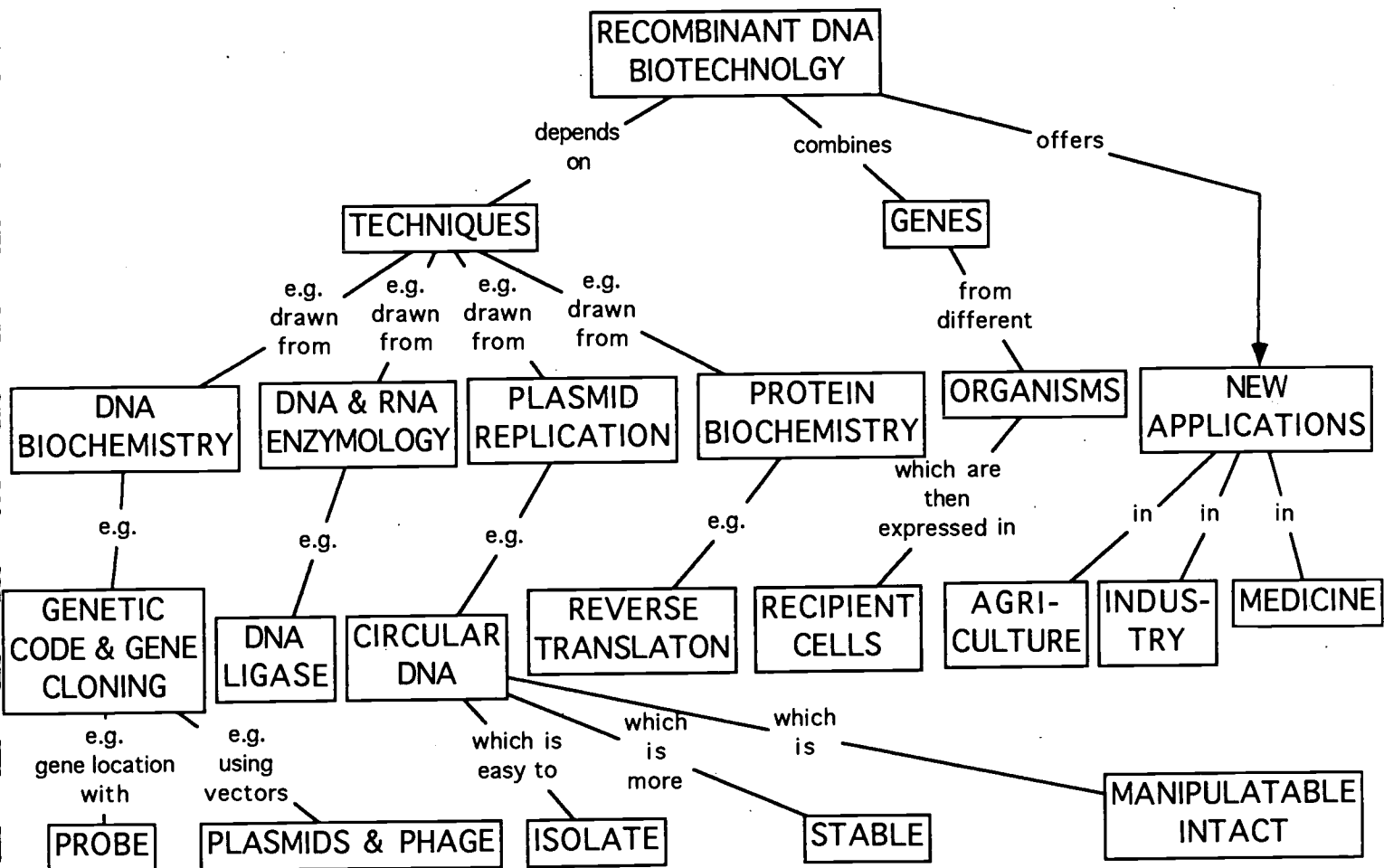
RECOMBINANT DNA

14

John Staczek

Overview

1. Composition of nucleic acids
2. Nucleic acids and genetic information
3. Extraction of DNA
4. Enzymes used to manipulate DNA
5. Designing recombinant DNA
6. Use of recombinant DNA



Recombinant DNA

Composition of Nucleic Acids

Nucleic acids are found in cells. There are two types of nucleic acids: ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). The building blocks of RNA and DNA are presented in Figure 14-1. RNA and DNA are comprised of nucleotides that are linked together in long polynucleotide chains. Nucleotides are made of phosphorylated ribose or deoxyribose sugars and purine or pyrimidine bases. The sugar ribose is found in RNA; the sugar deoxyribose is found in DNA. The purine bases are adenine (A) and guanine (G). The pyrimidine bases are cytosine (C), thymine (T), and uracil (U). The base uracil is found in RNA, whereas the base thymine is found in DNA.

Polynucleotide chains are formed when the phosphate group of the ribose or deoxyribose sugar forms a chemical bond with another ribose or deoxyribose sugar, respectively (see Figure 14-2). The ribose-phosphate-ribose (for RNA) or the deoxyribose-phosphate-deoxyribose (for DNA) linkage is the sugar-phosphate backbone of the polynucleotide chain. A polynucleotide chain containing only one sugar-phosphate backbone contains only one strand of polynucleotides and is called "single-stranded." "Double-stranded" molecules form when two single-stranded polynucleotide chains link together through hydrogen bonds that form between opposing purine-pyrimidine bases (see Figure 14-2). The resulting molecule has two sugar-phosphate backbones. In the double-stranded molecule, the matching of opposing purine-pyrimidine bases is very specific. Guanine always hydrogen-bonds with bases cytosine, which is called a *G-C pair*. The adenine-thymine pair (*A-T pair*) occurs only in double-stranded DNA. The A-U pair is found in double-stranded RNA or in hybrid molecules in which one strand is DNA (contains deoxyribose sugars and thymine), and the second strand is RNA (contains ribose sugars and uracil).

Nucleic Acids and Genetic Information

Polynucleotide chains contain the genetic information used to create proteins that direct biochemical pathways or that serve as structural components of the cell. The entire polynucleotide chain(s) that encodes genetic information for a life form is called its *genome*. Genomes may be relatively small, containing only several thousand nucleotides, or they may be as large as the set of human chromosomes that collectively contains a trillion nucleotides. A genome is made of either RNA or DNA, and

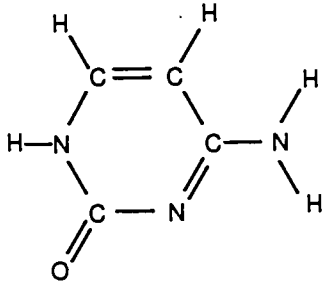
There are two types of nucleic acids, DNA and RNA, and each of them typically occur as long polynucleotide chains.

Nucleotides are made up of sugars and bases. DNA and RNA are polymers made up of nucleotides.

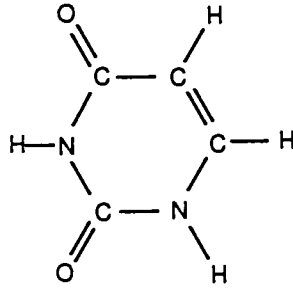
A polynucleotide chain can be single- or double-stranded.

Polynucleotide chains contain genetic information used to synthesize proteins.

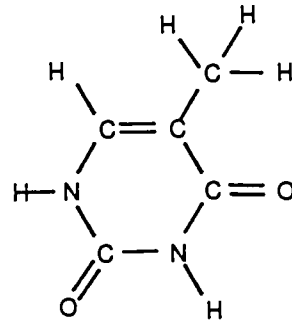
A. PYRIMIDINE BASES



Cytosine (C)

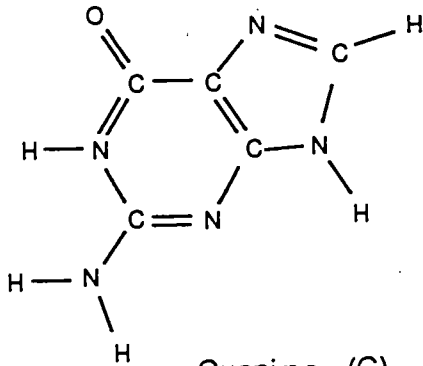


Uracil (U)

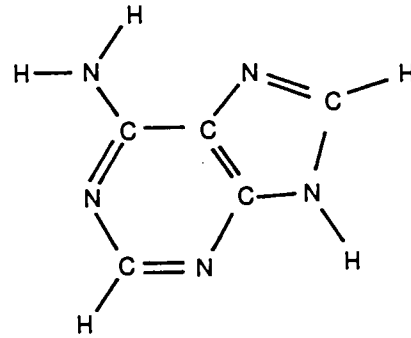


Thymine (T)

B. PURINE BASES

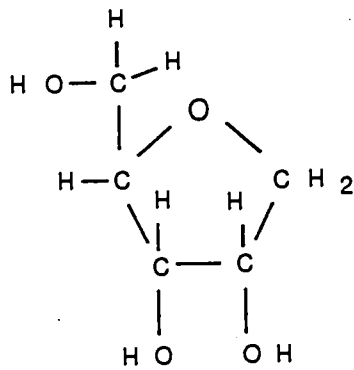


Guanine (G)

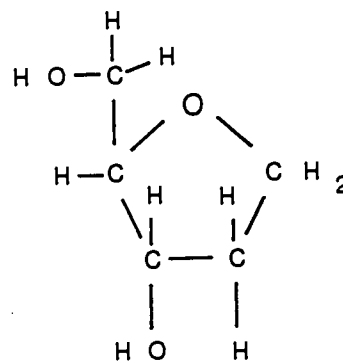


Adenine (A)

C. SUGARS



Ribose



Deoxyribose

Figure 14-1
Compositions of nucleotides.

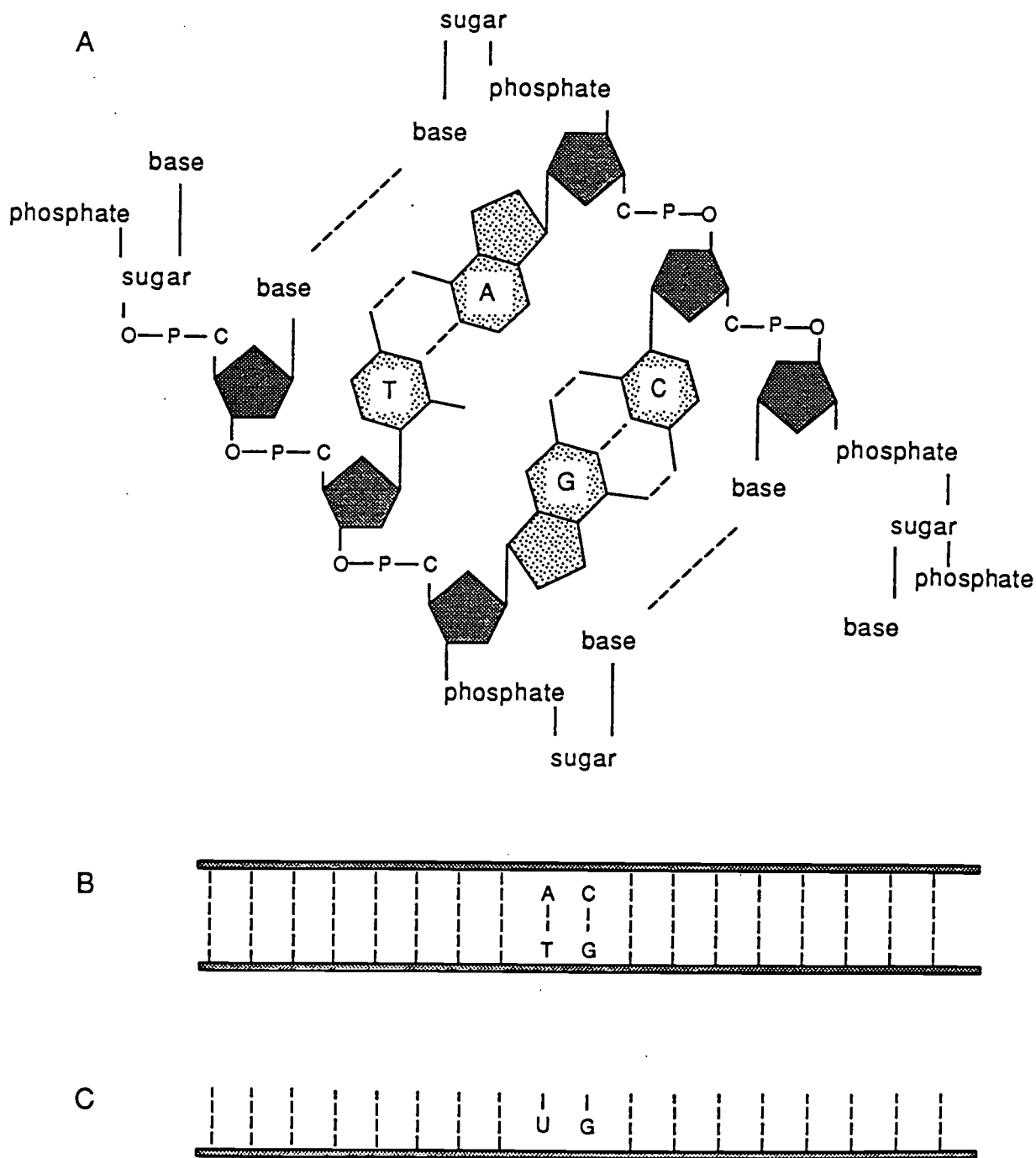


Figure 14-2
Polynucleotide chains (see text for description).

The term genome means the total genetic information carried by a cell or organism of interest.

The nucleotide sequence is the specific order of the nucleotides in a genome — this sequence encodes the genetic information and how it is expressed.

A gene is a fragment of DNA that controls a discrete hereditary characteristic, usually corresponding to a particular protein or RNA. The gene is a unit of information within the chromosome.

Transcription is the process by which one strand of DNA is copied into a complementary RNA molecule.

Messenger RNA (mRNA) carries genetic information from the gene to the ribosome where it determines the order of amino acids in the polypeptide. Messenger RNA is a result of transcription and functions in translation during protein synthesis.

Transfer RNA (tRNA) is a small RNA molecule, complementary in structure to mRNA, that transfers amino acids from the free state to the polymer form (the growing polypeptide chain) during protein making.

When the correct order of tRNAs is present, the amino acids will link together to form a protein — the order of the amino acids determining the structure of the protein.

it may be either single-stranded or double-stranded. The double-stranded DNA genome is the most common among all organisms, whereas single-stranded DNA genomes or single-stranded RNA genomes are found in some viruses. In cells with double-stranded DNA genomes, RNA molecules mediate the transfer of genetic information from the DNA molecules to proteins.

The order of the nucleotides in a genome is called the nucleotide sequence. It is the nucleotide sequence that encodes the genetic information and determines how the genetic information is expressed. Nucleotide sequences that encode information for proteins are called *genes*. Genes in a double-stranded DNA genome are not converted directly into proteins. Instead, the genes are converted into RNA copies.

The process of making an RNA copy is called *transcription*. During transcription, an exact copy of DNA is made. The RNA copy of a gene is called *messenger RNA*. The messenger RNA migrates to specialized organelles called the *ribosomes*. In the ribosome, the nucleotide sequence of the messenger RNA is translated into an amino acid chain by other RNA molecules called *transfer RNAs*. Each transfer RNA contains a series of three nucleotides, called an *anti-codon*, that pairs with a specific set of three nucleotides, called a *codon*, in the messenger RNA. In the ribosome, if the transfer RNA anti-codon matches the codon of the messenger RNA, then the transfer RNA remains associated with the ribosome. The next codon of the messenger RNA remains available until another transfer RNA matches it. When the next transfer RNA's anti-codon matches the next codon of the messenger RNA, it also stays in the ribosome. Attached to each transfer RNA is one specific amino acid. When two transfer RNAs, each containing its own specific amino acid, are next to each other in the ribosome, then a chemical bond can form between the two adjacent amino acids. After the amino acids are linked together, the first transfer RNA is released. The second transfer RNA remains behind, waiting for the next correct transfer RNA with its attached amino acid to match the next codon. When the correct order of transfer RNAs is present, the amino acids will link together to form a protein.

The order of the amino acids, in turn, determines the primary (linear) structure of a protein. The protein may subsequently fold into various shapes, such as a helix, a sheet or a globular mass. Proteins are the "work horses" of the cell. Proteins make enzymes that are catalysts for all biochemical reactions within the

Proteins are complex macromolecules composed of one or more polypeptide chains — each made up of a particular series of amino acids linked together by peptide bonds.

Promoter sequences are specific segments of DNA to which RNA polymerase binds to begin transcription of mRNA.

Polymerases are special enzymes that make DNA or RNA.

Double-stranded DNA genomes have enzymes that aid in the repair of damaged DNA.

cell. Proteins also combine with lipids, carbohydrates, and nucleic acids to form structural elements, such as membranes, organelles, or chromosomes, that maintain the integrity of the cell. Therefore, the integrity and function of a bacterial, plant, or animal cell (or a virus) are determined directly by the nature of the proteins in that cell. The nature of the proteins is indicated by its amino acids, and the type and order of the amino acids is directed by the sequence of the nucleotide codons found in the genes of its genome.

Some nucleotide sequences do not code for genes, but are used to regulate whether the genes will be made into messenger RNA or whether the genome will be replicated. Nucleotide sequences that regulate transcription are usually located next to genes and are called *promoter sequences*. Nucleotide sequences that start genomic replication are called *origins of replication*.

It is important that the genome of a cell is very stable and not subject to damage that would destroy the genetic information present in its nucleotide sequence. Otherwise, cell growth may be uncontrolled or possibly would not occur at all. Then useful genetic information would not be passed on to offspring.

However, damage to a genome does occur. For example, DNA is sensitive to ultraviolet (UV) radiation. That is why exposure to the sun may lead to skin cancer in humans or why bacteria have different growth patterns after exposure to UV irradiation. Fortunately, cells that have double-stranded DNA genomes also have enzymes that recognize the damaged DNA and repair the damage through elaborate biochemical pathways. These enzymes first remove the damaged portion of the double-stranded DNA and then fill in the resulting gap with new nucleotides placed in the same order as before the damage.

Because the repaired, double-stranded DNA has the same order of nucleotides as before becoming damaged, the genetic information has not been changed. Single-stranded DNA and single-stranded RNA genomes do not have these specialized enzymes and do not undergo repair as readily as double-stranded genomes. It is because of our ability to isolate and use the enzymes involved in double-stranded DNA repair that we can manipulate double-stranded DNA in the test tube.

Extraction of DNA

Laboratory procedures exist to extract DNA from viruses, bacteria, plants, and animal cells.

Extracted double-stranded DNA provides enzymes to make recombinant DNA.

DNA can be extracted from viruses, bacteria, animal, or plants. The method for disrupting cells varies and depends upon the nature of the cell wall. Animal cells are surrounded by a membrane comprised of lipids and proteins that can be disrupted when exposed to detergents. After the cell wall or membrane has been disrupted, the contents of the cells are subjected to centrifugation to separate the DNA from other cell constituents. This partially purified DNA is collected and extracted with organic solvents that denature proteins but leave the DNA intact. The denatured proteins no longer stick to DNA. The purified DNA is removed from the protein-DNA mixture by precipitation with ethanol. The precipitated DNA is resuspended in water that has small traces of salt and a pH of 7.8. If the procedure is followed with care, the DNA remains double-stranded, and the sugar-phosphate backbone remains intact. If the procedure is not carefully followed, the DNA may degrade into single-stranded polynucleotides or may fragment into small pieces if the sugar-phosphate backbone of the DNA polynucleotide chain breaks. The goal of the extraction procedure is to obtain the largest pieces of double-stranded DNA possible. Obtaining intact double-stranded DNA is very important because most, but not all, enzymes used to make recombinant DNA molecules come from cells that have a double-stranded DNA genome.

Enzymes Used to Manipulate DNA

Nucleases are enzymes that digest nucleic acids by breaking up the sugar-phosphate backbone of the polynucleotide chain via a process called hydrolysis.

Exonucleases are nucleases that remove nucleotides, one at a time, from the ends of entire DNA or RNA strands.

Enzymes that digest nucleic acids are called *nucleases*. Nucleases digest nucleic acids by breaking the sugar-phosphate backbone of the polynucleotide chain through a process called *hydrolysis*. Some nucleases only hydrolyze phosphate bonds located at the ends of double-stranded DNA. Such nucleases are called *exonucleases*. Digestion of double-stranded DNA molecules with exonucleases makes the DNA molecule progressively smaller (see Figure 14-3). Other nucleases interact with the internal regions of the double-stranded DNA molecule and hydrolyze the DNA at points that may be far from the ends of the DNA molecule. These nucleases are called *endonucleases*. Some endonucleases will hydrolyze double-stranded DNA at random points and, therefore, will digest the DNA into very small pieces. Other endonucleases recognize a specific order of nucleotides in the double-stranded DNA. These endonucleases are sensitive to the nucleotide order, and digest the

Endonucleases are nucleases that hydrolyze internal nucleotides to form smaller nucleotide chains.

The number of self-complementary (palindrome) sequences determines the number of times DNA will be cut by an endonuclease.

Restriction enzymes or nucleases act to protect bacteria from viruses by degrading viral DNA.

In the laboratory, restriction enzymes can digest all types of DNA.

Restriction enzymes cut double-stranded DNA to provide either blunted-ended or staggered cut fragments. Restriction enzymes break the hydrogen bonds that hold DNA together. These DNA fragments can connect with other single-stranded DNA fragments by re-forming the hydrogen bonds.

double-stranded DNA at the specific nucleotide sequence only. The specific nucleotide sequence that is recognized by some endonucleases is called a *palindrome* because the sequence is located in opposition and reverse order on both polynucleotide chains (see Figure 14-3). Sequence-specific endonucleases will hydrolyze the DNA molecule in or near the palindrome. These site-specific endonucleases usually cut a DNA molecule into a limited number of relatively large fragments rather than digest the DNA into very small pieces because the palindromic sequence that is recognized by the enzyme is found in the DNA only a few times.

A special group of sequence-specific endonucleases that are used in the test tube are obtained from bacteria. This special group of endonucleases represents many enzymes that are collectively called *restriction enzymes*. They are named restriction enzymes because these enzymes do not cut their own DNA molecules or DNA molecules from closely related bacteria, but they will cut DNA from other organisms that may get into the bacterial cell when the bacterial cell is taking up nutrients from its environment. Thus, in nature, the activity of these special endonucleases is "restricted" to the digestion of foreign DNA from other sources. Fortunately, these restriction enzymes lose their "restriction" in the test tube and digest all types of DNA including virus, bacteria, yeast, plant, or animal DNA. However, the DNA that has been purified from a cell and is in the test tube must still be double-stranded and must still have the palindromic sequence for a restriction enzyme to cut it.

Some restriction enzymes cut double-stranded DNA to produce fragments that are blunt-ended (see Figure 14-3). This is because the restriction enzyme digests the DNA by hydrolyzing the phosphodiester bond of opposing phosphates in each polynucleotide chain. Since the breaks are directly opposite, a clean cut or blunt end is made.

In contrast, other restriction enzymes produce staggered cuts. In these cases, the breaks in the sugar-phosphate backbone in each strand are not aligned directly across from each other. In the region between the breaks in the sugar-phosphate backbone, the DNA molecule is still held together by hydrogen bonds. The presence of the restriction enzyme causes the hydrogen bonds to break. The fragments produced are called "sticky" or "cohesive" ends because a part of the polynucleotide chain has become single-stranded and is available to interact with other single-stranded DNA molecules by re-forming hydrogen bonds.

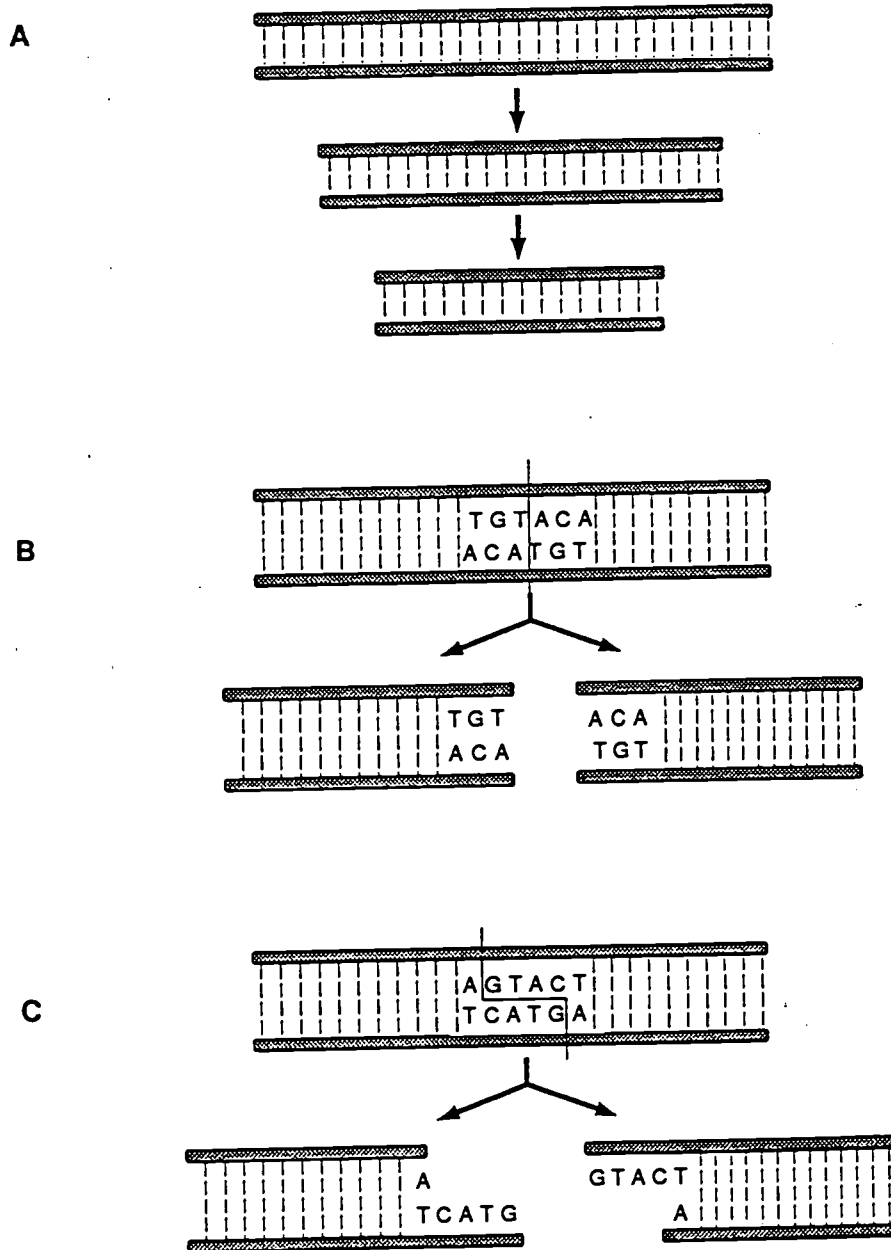


Figure 14-3
Nucleases.

DNA fragments can be reunited by a repair enzyme called ligase.

In much the same manner as when DNA in a cell becomes damaged, purified DNA that has been cut while in a test tube can be repaired. The ends of DNA fragments can be brought into close proximity and joined by special repair enzymes called *ligases*. Ligases take the DNA fragments and reconnect (or ligate) the sugar-phosphate backbone of the polynucleotide chains. The process of connecting two DNA fragments by rebuilding the sugar-phosphate backbone is called *ligation*. Ligases will not repair blunt-end DNA fragments as often as they will repair DNA fragments that have cohesive ends. Consequently, when DNA is manipulated in the test tube, scientists usually use restriction enzymes that produce cohesive ends on the DNA fragments. However, there are times when it is advantageous to produce DNA fragments with blunt ends.

When two DNA fragments with cohesive ends bind together or recombine, a new piece of DNA is created.

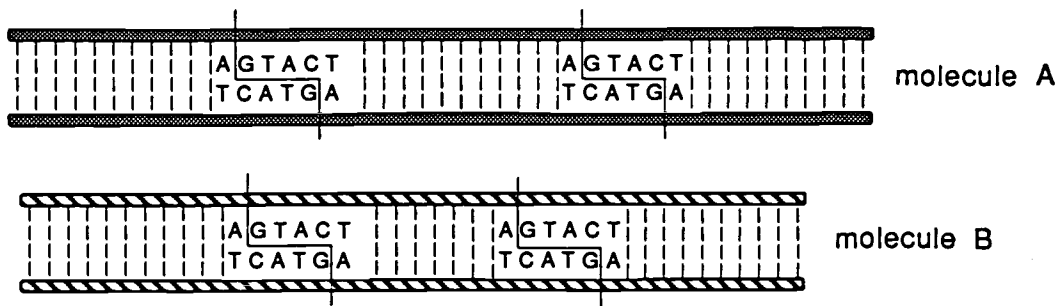
Restriction enzymes cut DNA at palindromic sequences. When purified DNA in a test tube is cut by one restriction enzyme, the DNA is cut only at the palindromic sequence. All of the resulting fragments contain identical ends. Because these DNA fragments are in solution, they are free to diffuse throughout the test tube. Consequently, the fragments get all mixed up. If the restriction enzyme used to cut the DNA is one that leaves the DNA with cohesive ends, then it is possible that two fragments may form hydrogen bonds between their single-stranded cohesive ends.

Recombinant (recombined) DNA molecule contains nucleotides that are in a different order than the original DNA.

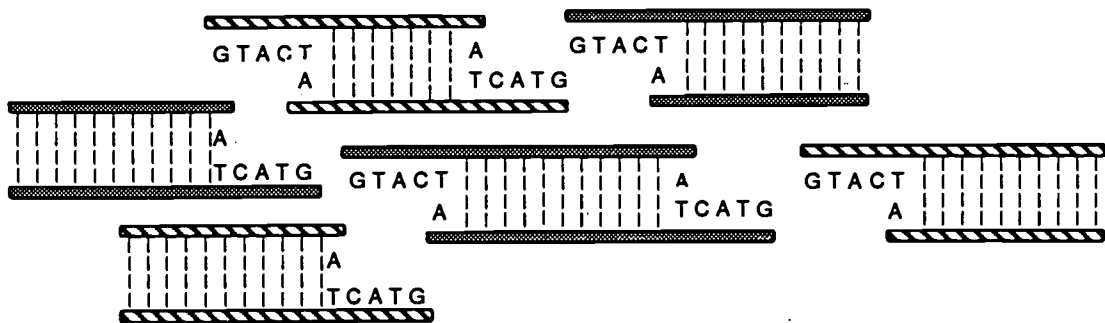
Recombinant DNA is any species of DNA that has been produced by subdividing natural DNA and then joining fragments to form new species of DNA. Recombination is often a natural process that can lead to the transmission of genetic traits, biological diversity, and the evolution of a species.

Since the DNA fragments are free to move about the test tube, any fragment can form hydrogen bonds through its cohesive end with any other fragment. The two fragments temporarily joined together through their cohesive ends may be from different parts of the original DNA molecule. Thus, the two fragments have recombined to form a new piece of DNA. This new DNA molecule contains nucleotides that are in a different order when compared to the original DNA molecule before it was cut by the restriction enzyme. If the nucleotide sequence is different, then the encoded genetic information in the new DNA molecule is also different with respect to the genetic information contained in the original DNA molecule. If a ligase were present, the ligase would repair the sugar-phosphate backbone, and the two DNA fragments would be permanently joined together. This new DNA molecule is called recombinant DNA (see Figure 14-4).

A. DNA molecules before digestion



B. DNA fragments after digestion with restriction enzyme



C. Recombinant DNA molecules

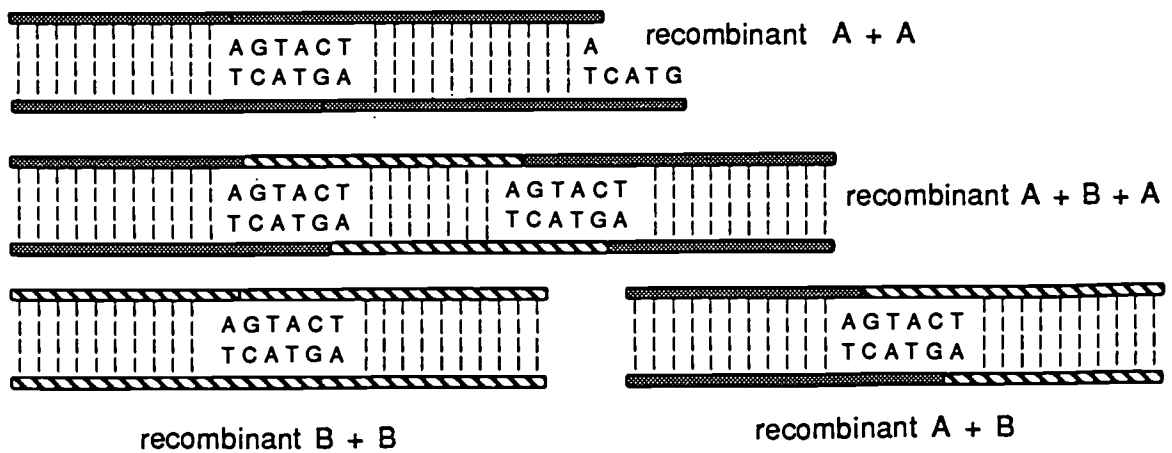


Figure 14-4

Formation of recombinant DNA molecules (see text for description).

Designing Recombinant DNA Molecules

Allowing DNA fragments to recombine randomly in the test tube will generate many recombinant DNA molecules. But in most cases, scientists want to design specific recombinant DNA molecules. To make specific recombinant DNA molecules, a procedure to analyze and select the DNA fragments of interest must exist. The procedure most commonly used is called *gel electrophoresis* (see Figure 14-5). In gel electrophoresis, DNA fragments are forced to migrate through an agarose gel. Agarose is a polysaccharide extracted from agar. An agarose gel is like the gelatin that is used in cooking. As gelatin or agarose harden, a semi-solid gel is made. Agarose gels are porous. The movement of molecules through the gel is dependent on the size of the molecule. Molecules that are very small will pass through most of the pores in the agarose gel. Most molecules have a slight, overall electric charge associated with them. When charged molecules are placed in an electric field, the molecules will move toward one of the two poles. Charged molecules will move toward a positive or a negative pole depending on whether their charge is negative or positive. Positively charged molecules will move toward a negative pole, and conversely, a negatively charged molecule will move toward a positive pole.

Gel electrophoresis is used in generating specific recombinant DNA molecules.

DNA is negatively charged and will migrate toward the positive pole during gel electrophoresis. Large DNA fragments migrate much more slowly through the gel than smaller fragments. DNA fragments of the same size migrate together at the same rate. If a mixture of restriction enzyme-cut DNA is placed into a hole (called a well) in the agarose gel, and an electric field is applied across the gel, the DNA fragments migrate toward the positive pole. As the DNA fragments migrate through the gel, they separate according to their sizes. The separated DNA fragments are visualized after the gel is treated with a special chemical that fluoresces when exposed to UV light. DNA fragments of the same size migrate together and form a visible band (see Figure 14-5).

As DNA migrates through the gel, DNA fragments separate according to size.

The rate at which DNA migrates through the gel is affected by its size (i.e., the larger the DNA fragment the slower the migration rate).

To make a recombinant DNA molecule, the bands of DNA are cut out of the gel. Next, the DNA is separated from the agarose, usually by melting the agarose. When the agarose is melted, water is added to dilute the agarose. The diluted agarose will not form a gel. The DNA is released from liquefied agarose and subsequently precipitated from the solution. The precipitated DNA is resuspended. This resuspended DNA fragment is usually mixed with another purified DNA fragment

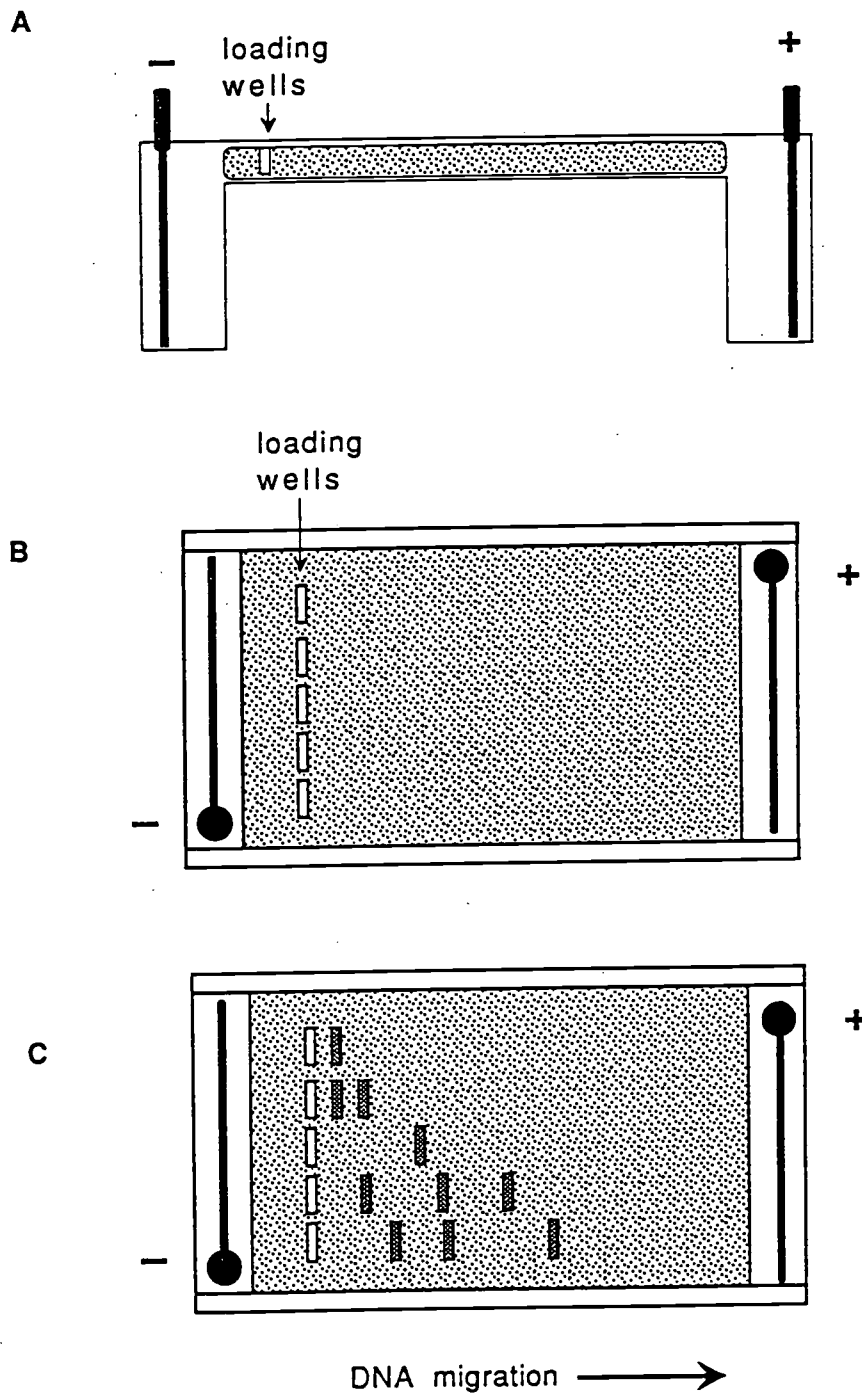


Figure 14-5
Gel electrophoresis.

Cohesive ends of two fragments of precipitated DNA from gel electrophoresis are combined to form recombinant DNA

that was digested with the same restriction enzyme and isolated by gel electrophoresis. The cohesive ends of each fragment permit hydrogen bonds to form between the two different fragments to form recombinant DNA molecules. These recombinant DNA molecules are incubated with ligase to join together the sugar-phosphate backbones of the polynucleotide chains.

When two different DNA fragments containing cohesive ends are put into solution and allowed to recombine, the fragments will recombine randomly to produce several different recombinant molecules (see Figure 14-5). These different recombinant molecules will be made of different ratios of each of the two fragments. If two fragments, for example fragment A and fragment B, are mixed together, the possible recombinant DNA molecules include, but are not limited to: fragment A + fragment B; fragment A + fragment A; fragment B + fragment B; fragment A + fragment A + fragment A; fragment A + fragment B + fragment A; etc. Gel electrophoresis is used to separate the recombinant DNA molecules. Each combination of fragments migrates according to its size. To obtain a specific recombinant DNA molecule, the appropriate band is identified, cut out from the gel, and separated from the agarose.

Recombinant DNA Molecules Represent RNA or DNA Genomes

The procedures described above use enzymes that work on double-stranded DNA. Therefore, recombinant DNA molecules are usually made from organisms that have double-stranded DNA genomes. Genomes of bacteria, yeast, plant, and animal cells are typically made of double-stranded DNA and are used extensively to make recombinant DNA molecules. Virus genomes, however, may be DNA or RNA, but not both. If a genome is made of RNA, then it must be converted to DNA (Figure 14-6). A special enzyme, called *reverse transcriptase*, will make a DNA copy of an RNA genome. This DNA copy has exactly the same genetic information as the RNA genome, but the nucleotide sequence has thymine instead of uracil, and the sugar-phosphate backbone has deoxy-ribose sugars instead of ribose sugars. The DNA copy of an RNA genome is called *complementary DNA*. Complementary DNA is single-stranded. Since restriction enzymes digest double-stranded DNA only, another enzyme, called *DNA polymerase*, is used to make a DNA

The enzyme reverse transcriptase transcribes a RNA genome into a complementary DNA molecule.

Complementary DNA is single-stranded and requires DNA polymerase to make it double-stranded.

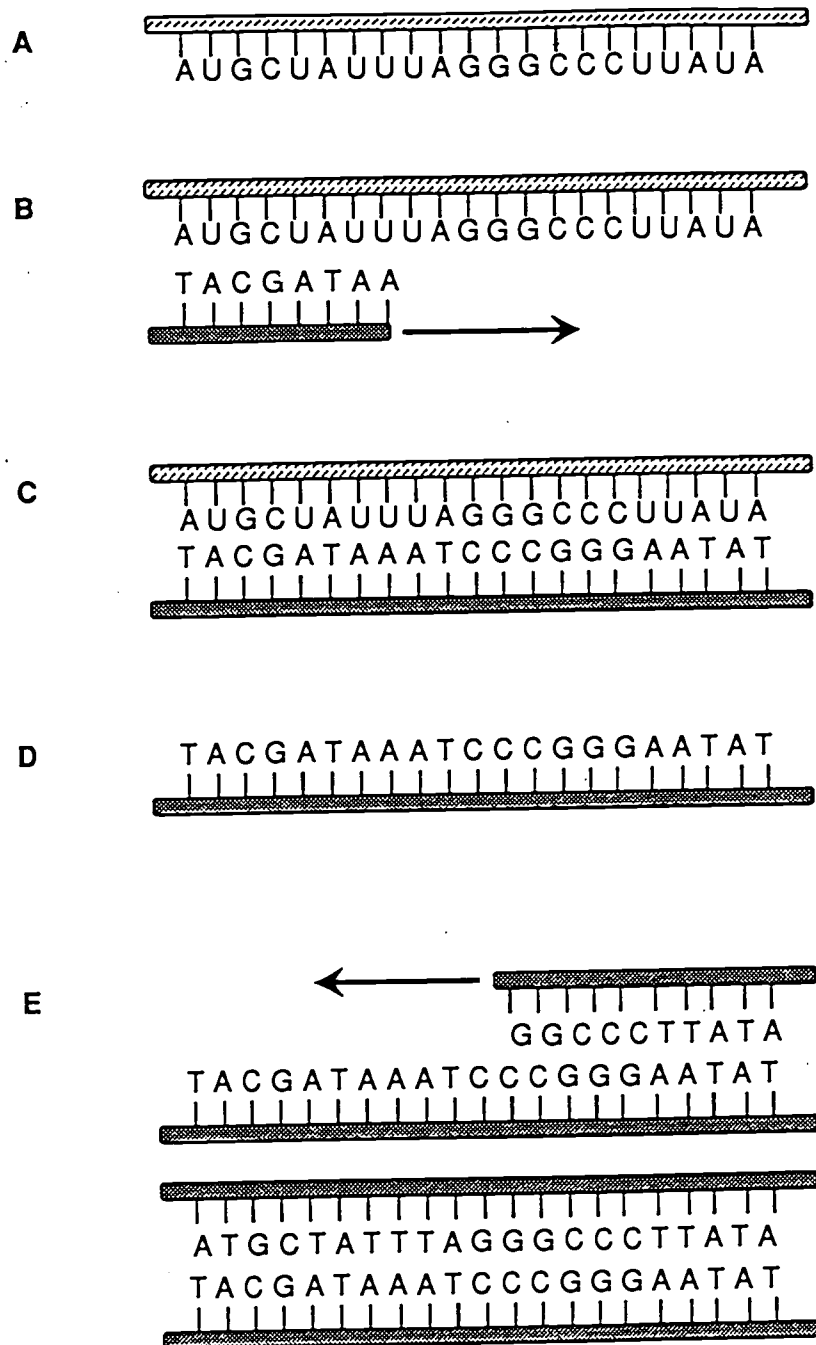


Figure 14-6
Making complementary DNA.

copy of the complementary DNA. This DNA copy remains hydrogen bonded to the complementary DNA. Therefore, the final DNA molecule is double-stranded. This double-stranded DNA molecule is an exact copy of the nucleotide sequence of the RNA genome and can be used to make recombinant DNA molecules. This entire procedure can be done in the laboratory.

Bacterial Plasmids

A plasmid is used to obtain large quantities of recombinant DNA.

A plasmid is a small DNA molecule that replicates independently of the genome and is used as a vector for DNA cloning.

Plasmids are fragments of the DNA that replicate within a bacterial cell.

When a recombinant plasmid is reinserted into a bacterial cell, it can replicate and direct synthesis of large amounts of protein from its DNA.

Recombinant DNA molecules are made in a test tube. However, the quantity of recombinant DNA molecules is often very small. It is easier to work with larger quantities of recombinant molecules. To obtain larger quantities of recombinant DNA molecules, scientists often place their recombinant DNA molecules into special DNA molecules called *plasmids*. In nature, plasmids are found in bacterial cells. The genetic information of all bacterial cells is encoded in its DNA genome. Many bacterial cells have additional genetic information that is encoded in DNA that is not part of its genome. These extra chromosomal pieces of DNA are plasmids.

In bacteria, plasmids are double-stranded DNA molecules that contain genetic information to make protein molecules which help the bacteria become resistant to antibiotics. Plasmids are unique pieces of DNA because they replicate in the bacterial cell so that one bacterium contains up to 200 plasmid molecules. Plasmids can be extracted from the bacterial cell. Intact plasmids are double-stranded and can be digested with the restriction enzymes or ligated with ligases. Plasmids that contain DNA from other bacteria, yeast, animal, or plant cells are considered *recombinant plasmids*. These recombinant plasmids are small DNA molecules that are easily identified in agarose gels after gel electrophoresis. Recombinant plasmids can be reinserted into bacterial cells where they will replicate and direct the synthesis of large quantities of protein from their DNA. Scientists take advantage of these unique properties of plasmids and frequently use the plasmids to make large quantities of recombinant DNA molecules or proteins.

Putting Recombinant DNA Molecules into Cells

Recombinant plasmid DNA molecules by themselves do nothing. The DNA molecule is simply a good storage place for genetic information. For genetic information to be used, cellu-

lar enzymes and structures called *ribosomes* are required to convert the information in the nucleotide codons into the amino acid chains that make up proteins. To be converted into useful proteins, DNA must be inside a cell or in a test tube containing all of the constituents of a cell. The conversion of nucleotide codons into proteins in the test tube is not yet a very practical way of obtaining proteins. It is more efficient to place the recombinant plasmid DNA molecules into cells.

Once recombinant DNA is injected into a recipient cell, or manipulated to enter the cell membrane, it behaves like native DNA.

Recombinant DNA molecules are not easily taken up into bacterial, plant, or animal cells. The recombinant DNA can be injected directly into animal cells, as into frog oocytes, for example, or treated with a compound called *calcium phosphate*. The recombinant DNA must be treated with calcium chloride or subjected to an electric field if the target cell is a bacterium. In addition, the recipient cells have to be treated to make their cell membrane or cell wall permeable to the recombinant DNA. When the recombinant DNA is in the cell, it behaves just like native DNA. The integrity of the cell membrane or wall returns to normal, and the cell or bacterium is subsequently grown to large quantities.

The nucleotide sequence of recombinant DNA is identical to the nucleotide sequence of the original DNA.

If all of the procedures are followed carefully, the nucleotide sequence of the recombinant DNA remains identical to the nucleotide sequence of the original DNA. In the cell, the nucleotide codons of the recombinant DNA molecule direct the order of the amino acids for a protein just like the codons in the original DNA would. The protein made using the genetic information of the recombinant DNA molecule is identical to the protein made from the original DNA molecule.

Occasionally, however, the protein encoded in a recombinant DNA molecule is not be the same as the protein encoded in the native DNA molecule because nucleotides were lost or somehow damaged during the cutting of the native DNA or processing of the fragment. This change in the order or number of nucleotides is called a mutation. Mutations in DNA molecules cause the amino acid order of proteins to deviate from normal. The resulting mutated proteins behave abnormally.

Uses for Recombinant DNA Molecules

Analysis of Nucleotide Sequences

Plasmids are used to make large quantities of recombi-

DNA sequencing is the process of determining the nucleotide sequence of the nucleic acids in the DNA sample of interest.

nant DNA. One use for large quantities of recombinant DNA is the analysis of the nucleotide sequences. If all the procedures for making a recombinant DNA molecule are done correctly, the resulting recombinant DNA is an exact copy of the original DNA obtained from the cell. Thus, if the nucleotide sequence of the recombinant DNA is determined, the nucleotide sequence of the original DNA will be known. The process for determining the nucleotide sequence of a nucleic acid is called *sequencing*. If the nucleic acid is DNA, then the sequencing is called *DNA sequencing*. DNA sequencing is a complicated process that may take several days or even weeks to do. Many viral and several bacterial genomes have been successfully sequenced. Scientists have begun to sequence the entire human genome. It is predicted that this effort will take many years.

Determining the Functions of Proteins

Knowing the exact order of nucleotides in DNA is important because genomes contain nucleotide sequences that encode genetic information to make proteins and to control the accessibility of that information. A series of codons that supplies the information to order the amino acids for protein synthesis is called a *gene*. Control regions, called *promoters*, are nucleotide sequences that precede the gene and determine when the gene will be used.

The study of mutations allows scientists to understand how promoters affect transcription from DNA.

Genes and promoters are often ligated into recombinant DNA plasmids so that they can be sequenced. Once the correct order of the nucleotides is known, the order can be changed. The altered nucleotide sequence is called a *mutation*. The ability to make mutations is a very powerful tool for studying how a promoter affects transcription from the DNA. Altering the nucleotide sequence of a promoter will determine whether a protein is made or not made. The ability to mutate genes changes the order of amino acids in a protein and alters the function of that protein. When a protein is not functioning in a cell because the protein is not made or because it is made incorrectly, the cell behaves differently. By comparing normal cells to cells containing mutated proteins, scientists can deduce the role of the normal protein.

Making Large Quantities of Proteins

Promoters are generally cell specific. This means that a promoter taken from a bacterial genome will not work in animal cells; a promoter taken from an animal genome will not work in plants, etc. However, animal genes placed behind a

Purified proteins can be used in biochemical studies, or used as drugs to treat or prevent human disease.

bacterial promoter on a plasmid will be made into proteins if the recombinant DNA is subsequently placed into a bacterial cell. The recombinant plasmid will replicate itself to high numbers (100-200 per bacterial cell), and each plasmid will make hundreds of protein molecules. If a bacterial culture has billions of bacteria in one milliliter of culture, each of which contains hundreds of copies of the same recombinant plasmid, then relatively large quantities of protein can be made. The proteins can be purified and used for biochemical studies in the laboratory or used as drugs (such as insulin) or vaccines (such as Hepatitis B vaccine) to cure or prevent disease.

Correcting Genetic Defects

Recombinant DNA may be useful in correction of human genetic defects.

Some human diseases are the result of naturally occurring mutations in the human genome. The cell has mechanisms using damage and repair enzymes to correct mutations. But sometimes a mutation does not get corrected. Some of these mutations reside in the genomes of parents and can be genetically passed on to their children. If the mutation can be identified, then recombinant DNA molecules containing a corrected version of the mutated nucleotide sequence can be made. The recombinant molecules can be transferred into human cells in the laboratory. It is hoped that these same corrected genes can be successfully placed into humans.

Destroying Cancer Cells

Recombinant DNA from viruses may be effective in eradicating certain cancer cells.

Viruses can be made to carry recombinant DNA molecules. These recombinant viruses can be altered genetically to infect only specific cells such as cancer cells. These recombinant viruses can also contain genetic information that would make the infected cells susceptible to certain drugs. Cancer cells infected with the recombinant virus could be selectively killed when the drug is given to a cancer patient.

Making Recombinant Fruits and Vegetables

Recombinant DNA from plant cells expressing desired plant characteristics can be introduced directly into recipient plant cells to produce more healthful fruits and vegetables.

Farmers have always been developing crops that are disease resistant, easy to grow, and easy to harvest. Usually, pollen from a plant that has some desirable characteristics is used to fertilize another plant that has other desirable characteristics. The offspring of successful pollination might or might not have a combination of all the desired characteristics. Recombinant DNA molecules containing the genes for the desired characteristics can be introduced directly into the cells of the plant

or the germ line cells of the plant and be passed on to offspring by cuttings or pollination.

Recombinant DNA Molecules Are Safe to Make

During the early years following the production of the very first recombinant DNA molecule, there was much concern as to whether recombinant DNA molecule could “escape” from the laboratory and “invade” living organisms. Committees of scientists and lay people were formed and guidelines for the making of recombinant DNA molecules were established. These guidelines imposed very stringent biological and laboratory conditions for making recombinant DNA molecules. Fortunately, during the years that the guidelines were in effect, it became apparent that many of the fears concerning recombinant DNA molecules were unfounded. Consequently, most forms of recombinant DNA research are no longer regulated.

Questions Most Commonly Asked by Students

Question 1: Which viruses have RNA genomes?

There are several. There are two types of RNA genomes found in viruses. One type of RNA genome needs to be transcribed into messenger RNA for the genetic information of the virus to be translated into protein. An example of this type of RNA genome is the influenza virus. The second type of RNA genome can be directly translated into protein; thus the viral genome is also a messenger RNA. An example of this type of RNA genome is poliovirus.

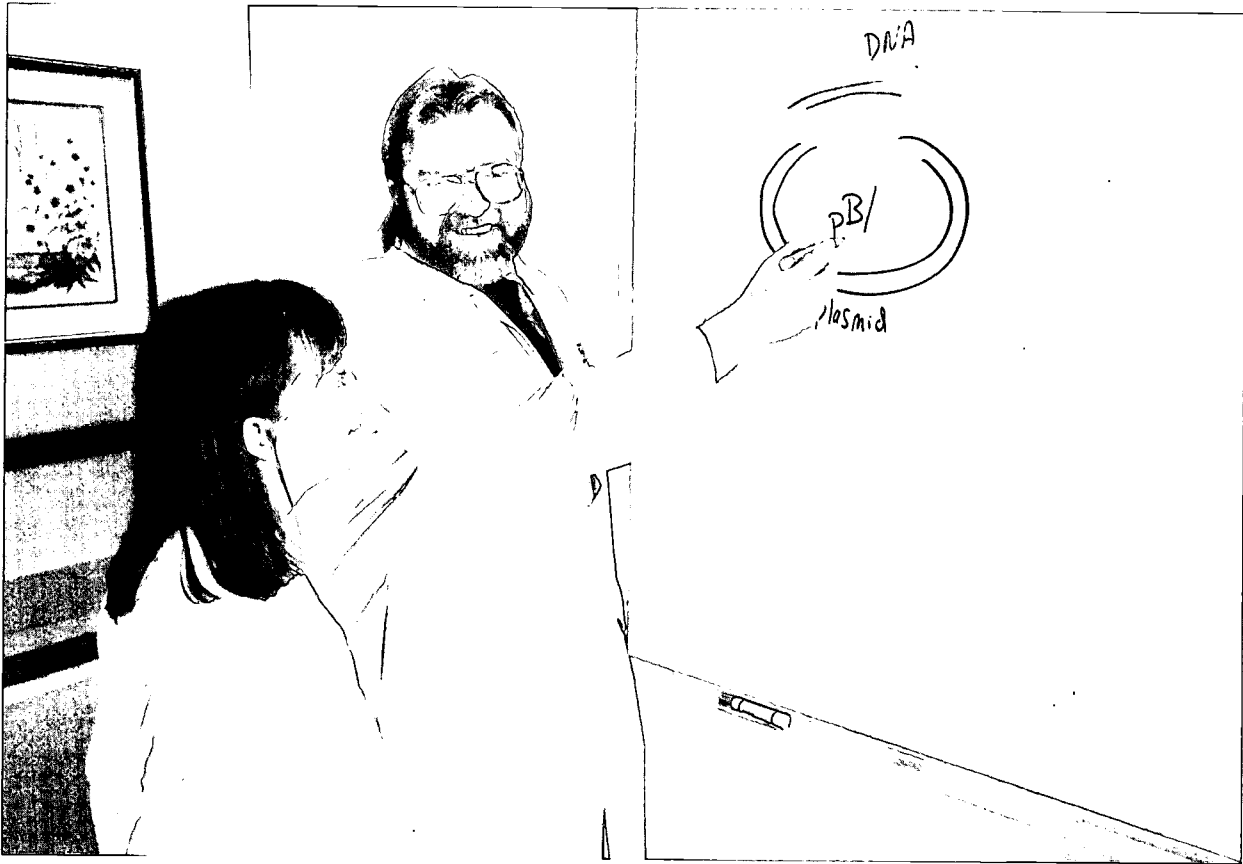
Question 2: Why can genes from animals or plants be transcribed and translated into proteins in bacteria?

The biochemical pathways for making messenger RNA and for translating the messenger RNA into proteins are very similar. The nucleotides and amino acids are the same. There are slight differences, however, between animal, plant and bacterial ribosomes, so the appropriate animal, plant or bacterial promoter to which the ribosome attaches on the messenger RNA must be present.

A major difference in proteins made in bacteria versus proteins made in animal cells is that animal proteins are modified with sugars, phosphate and lipids, whereas bacterial proteins are not. The bacterial cells do not have the ability to correctly modify animal proteins as they are being synthesized in a bacterial cell. For many purposes, such as sequencing, it is not important if the animal or plant protein made in the bacterial cell is modified. If the modifications are important, then the recombinant DNA must contain a promoter from an animal cell or a virus that infects animal cells and be placed into an animal cell, not a bacterial cell.

Question 3: Can any bacteria be used to grow plasmids containing recombinant DNA?

In theory, the answer is yes. However, only certain bacteria are used. The most widely used bacterium is a specialized Escherichia coli. This strain is special because it has been genetically manipulated so that it cannot survive without special conditions for growth. These special conditions can be supplied only in the laboratory.



Dr. John Staczek explains recombinant DNA to Kim A. Garko, M.S., a graduate student in the Department of Microbiology and Immunology at LSU Medical School in Shreveport, LA.

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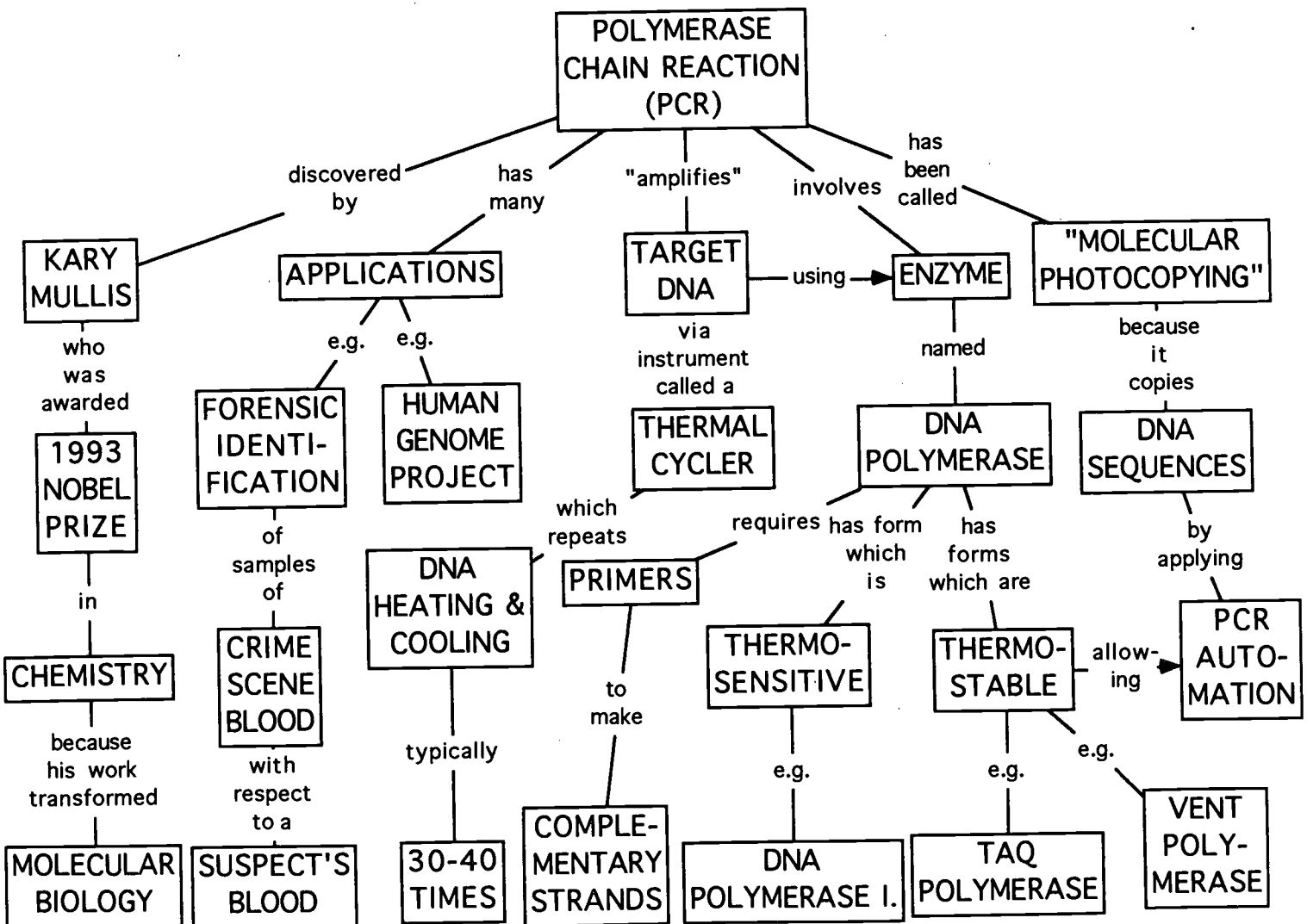
THE POLYMERASE CHAIN REACTION

15

Lynda A. Britton

Overview

1. History of PCR
2. Principles of DNA chemistry
3. Basic PCR procedures
4. Applications of PCR



The Polymerase Chain Reaction

Introduction

The polymerase chain reaction (PCR) is a process that duplicates selected segments of DNA.

The polymerase chain reaction (PCR) has become a mainstay of modern molecular biology. It is the *in vitro* enzymatic amplification of DNA by DNA polymerase to make millions or billions of copies of a segment of nucleic acid. PCR's ability to amplify DNA segments is based upon the properties of DNA itself. PCR has been called *molecular Xeroxing*^(R) and is described as a process in which a single molecule of the genetic material (e.g, DNA) can be duplicated into 100 billion molecules in a few hours (see Figure 15-1). This chapter was written to help classroom educators teach students the basic principles of this remarkably simple procedure that has revolutionized the field of molecular biology. It is not intended to describe the myriad of technical details required to make it work in the laboratory.

History of PCR

The origin of PCR was in 1983.

PCR was discovered by Kary Mullis on a Friday night in 1983 on California Highway 128 in Mendocino County while he was driving to his cabin in the woods. He was mentally working on the problem of how to determine the identity of a specific nucleotide in a gene in order to diagnose a particular genetic disease. He visualized the components of DNA as appearing like Tinker Toys^(R) and mentally rearranged them. A flash of insight gave him PCR, the answer to this problem. Using the initial idea of PCR, he realized that specific DNA sequences could be duplicated into millions of identical copies in the laboratory.

PCR has become an important tool for the molecular biologist.

Mullis was a scientist working for the Cetus Corporation of Emeryville in Northern California when he discovered PCR. The investigators at Cetus were not impressed with the idea of PCR until it was presented in 1986 at the Cold Spring Harbor 51st Symposium on Quantitative Biology, *The Molecular Biology of Homo Sapiens* (organized by James Watson, who is one of the scientists who discovered the structure of DNA in 1953). Following this conference, investigators worldwide began to appreciate PCR and its potential for solving many of the problems associated with molecular biology. Since then, PCR has shown to be an indispensable tool for the molecular biologist. PCR rapidly transformed the way scientists do molecular biology. With-

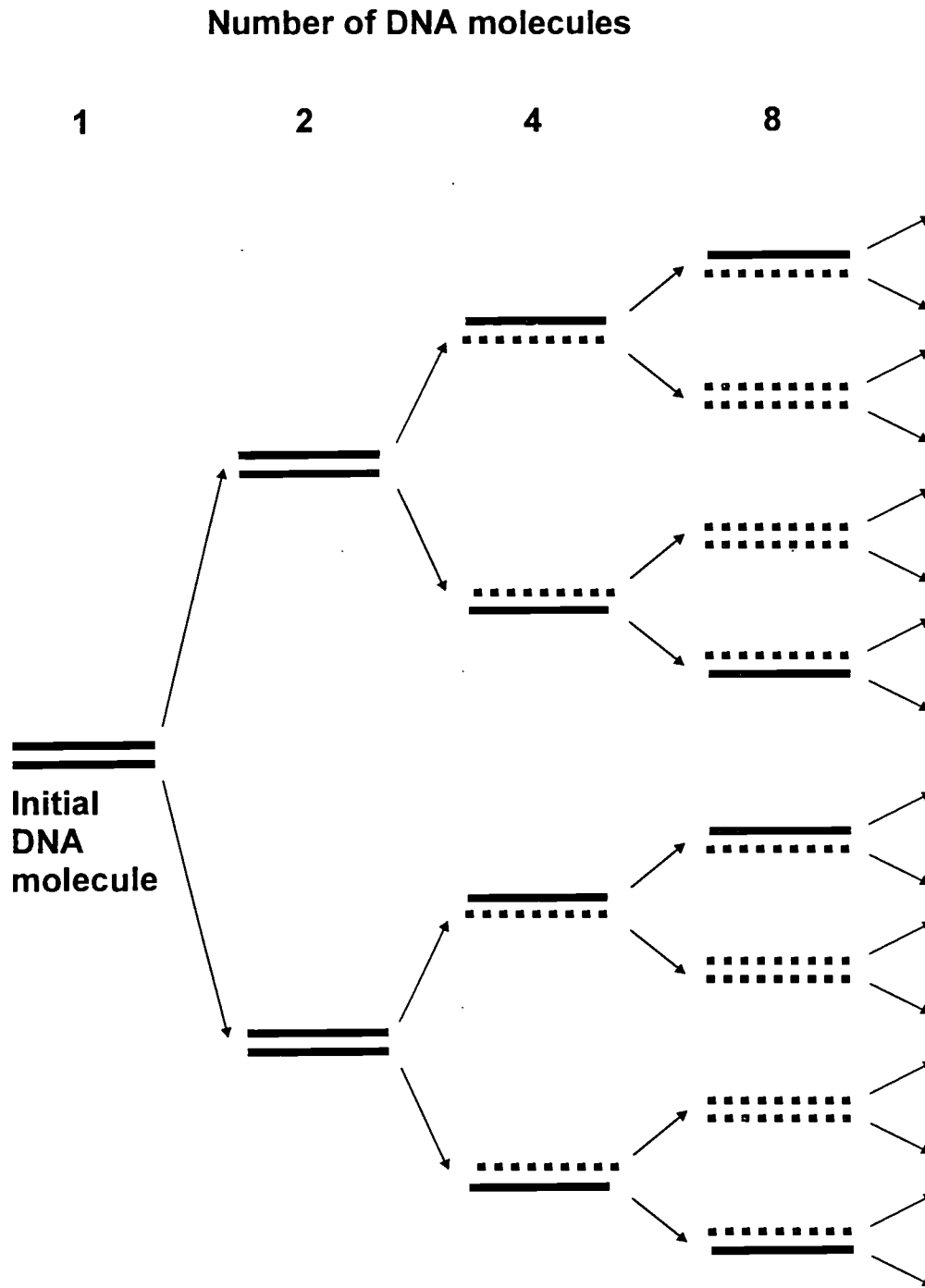


Figure 15-1
PCR.

Kary Mullis, who discovered PCR, published his findings in 1985 in Science.

out PCR, for example, the Human Genome Project would overwhelm scientists, cost millions of dollars more, and take many years longer to complete. Mullis first published his findings in December 1985 in *Science*. Cetus was assigned the patent for PCR, while Mullis was given \$10,000. In 1993, Cetus sold the patent for PCR to Hoffmann-LaRoche for \$300 million, while Mullis was awarded the Nobel Prize for Chemistry in the same year for his discovery of PCR.

Principles of DNA Chemistry

DNA is a double-helix molecule formed from two coiled complementary strands made up of nucleotides.

Every living organism has the hereditary machinery to make copies of itself. The spiral-shaped DNA molecule is largely responsible for this ability to reproduce. DNA consists of two complementary strands coiled around each other to form a twisting ladder called a *double helix*. The strands are made up of varying sequences of chemical groups called *nucleotides*. The backbone of the double helix consists of deoxyribose and phosphate, while either two purine bases or two pyrimidine bases are arranged like rungs in a ladder. The two purine bases are adenine and guanine, while the pyrimidine bases are cytosine and thymine. These bases are arranged like rungs on a ladder. They are attached to the backbone on one end and to their complementary base in the center where they are held together with hydrogen bonds. Adenine pairs with thymine, and cytosine pairs with guanine on the double helix. Small segments of genetic information can be amplified with PCR to make a large quantity of identifiable and analyzable material. PCR is based upon the ability of DNA to replicate itself in a semiconservative manner. DNA polymerase is one of the enzymes that duplicates the DNA when a cell divides so that there are two exact copies of the chromosomes, one for each new cell. DNA polymerase causes the two strands to separate at the bases and then adds new bases to each strand resulting in the making of a complementary copy of the original DNA.

Small amounts of genetic information can be amplified with PCR to aid in its identification.

DNA polymerase polymerizes nucleotides to form new DNA strands.

Heat breaks hydrogen bonds between bases and the DNA becomes single-stranded.

When DNA is heated to over 90° C, the hydrogen bonds between bases break and the DNA becomes single-stranded. Thermal energy from heating increases molecular motion that weakens the hydrogen bonds. The two strands come back together (reanneal) when the temperature is lowered to 55° C or less. The backbones of the DNA run in opposite directions; the five prime (5') and three prime (3') ends are named for the ori-

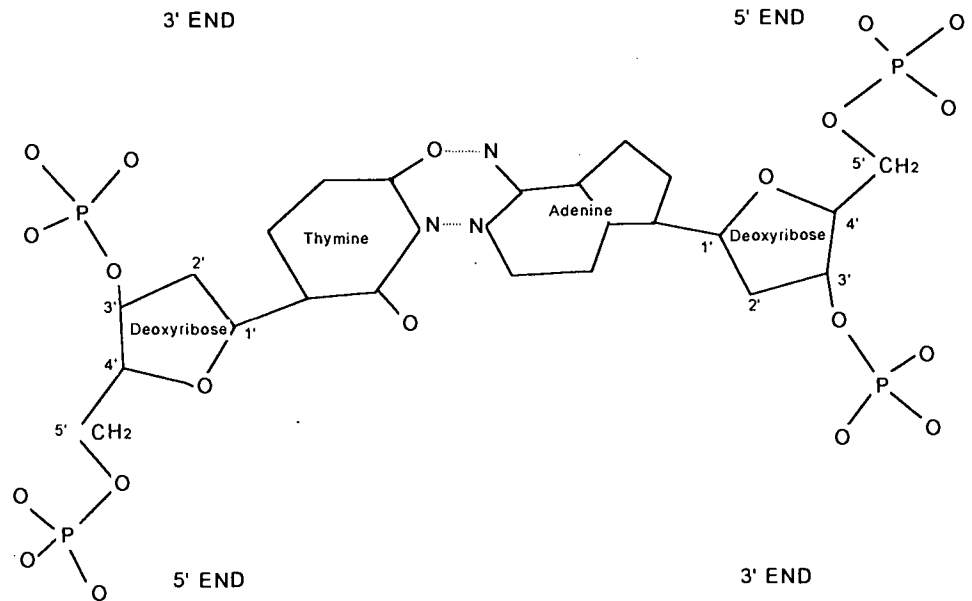


Figure 15-2

entation of the 5' and 3' carbon atoms of the deoxyribose rings (see Figure 15-2 above)

Basic PCR Procedure

The basic PCR procedure is completed in three steps. The first step includes the extraction of DNA from the cells. This is followed by heating the DNA to about 95° C to melt it into single strands. Then the DNA strands are cooled to about 50° C so that primers flanking the DNA to be amplified can anneal to the single strands. The DNA to be amplified is called the *target*. Primers are short oligonucleotides complementary to the target and are necessary because the DNA polymerase cannot work without a starting point. DNA polymerase adds new nucleotides only at a specific site that includes the molecules of both strands, and it can only move in one direction. These primers provide the specificity of PCR by bracketing only the target. In the third step, the DNA polymerase extends the primers by adding nucleotides to make a complementary strand of DNA that includes the target. This process is repeated thirty or forty times, and the number of copies increases exponentially. Theoretically, one cell can pro-

An oligonucleotide is a polynucleotide of low molecular weight (a short strand).

vide a billion copies of the target in 30 cycles. In actual laboratory practice, the maximum is never achieved, and more cycles or more targets are typically required.

Target

The target is the sample DNA, RNA, or gene to be amplified.

The target may be a gene or a segment of DNA or RNA that interests the investigator performing the PCR procedure. If RNA is used, it must be converted to DNA by reacting it with the enzyme reverse transcriptase. The target is a unique sequence of nucleotides from 100 to 1,000 base pairs long. A target of 200 to 500 base pairs is considered optimal size. This number of bases is required for fast replication and specificity of the reaction. Most of the sequences of the target must already be known in order to choose unique primers.

Primers

The two primers are bound to the opposing strands in the DNA at sites flanking the targeted base pairs.

The primers are short, single-stranded, oligonucleotides that bracket the target. Primers are synthesized using an automatic procedure that is relatively inexpensive. They can be made to the scientist's requirements or purchased from a supplier. Two sets of primers are required. One primer is a copy of a short section of the coding strand of DNA at the 5' end of the target and the other is a copy of the noncoding strand at the opposite 5' end of the target. Primers are usually 20 to 30 nucleotides long and must not be complementary to each other. They also must be unique and only anneal to the target; therefore, most of the sequences of the DNA in the target must be known. The size of the primer must be suitable for efficient amplification, but long enough to anneal only to the target. Because they are added in excess and are short, when the mixture is cooled to about 50° C during the PCR procedure, they anneal to the target DNA. Primers anneal first before the long strands of the target can come back together. They provide a starting point for the DNA polymerase enzyme to synthesize a second strand complementary to the target.

Primers allow DNA polymerase to synthesize and duplicate a copy of the target.

DNA Polymerase

Taq polymerase became the enzyme of choice to use with PCR, because it does not denature at high temperatures.

When PCR was first developed, the Klenow fragment of *Escherichia coli* DNA Polymerase I was used to extend the primers. This polymerase was tedious to use because it had to be added to the mixture at each cycle, since its activity is destroyed at temperatures above 90° C. *Taq* polymerase made it possible to

When the target base is adenine, *Taq* polymerase adds thymine; when the target base is guanine, the polymerase adds cytosine.

fully automate the procedure because it is not denatured at high temperatures. It works repeatedly during the heating and cooling stages of PCR. This is because it was originally isolated from *Thermus aquaticus*, a thermostable eubacterium found in hot springs at Yellowstone National Park. *Taq* polymerase was named the first "molecule of the year" by *Science* in 1989. This thermostable polymerase synthesizes new DNA from the 3' end of the primer by adding deoxynucleotide triphosphates that are complementary to the target. In other words, when the target base is adenine, *Taq* adds thymine. When the target is guanine, it adds cytosine. The optimum temperature for *Taq*'s enzymatic activity is 72° C, which is why the temperature is raised to 72° C during the third step of the PCR procedure.

The DNA thermostable polymerase named VENT offers the advantage of correcting mistakes made during the amplification process.

Taq polymerase works quickly, adding about 60 nucleotides per second at 72° C. It moves in only one direction, from the 5' to the 3' direction and has no proofreading ability. To work properly, magnesium is required by the enzyme as a cofactor. Maintaining its concentration is one of the technical problems of PCR. *Taq* is provided by a strain of genetically engineered *Escherichia coli* which has been cloned to produce this enzyme. *Taq* is commercially available from Perkin-Elmer. There is another thermostable DNA polymerase available called VENT. It was isolated from *Thermococcus litoris* bacteria that live near hot ocean vents. VENT has 3' to 5' exonuclease (proofreading) activity and should have a lower misincorporation rate since it can correct any mistakes it makes.

Master Mix

Amplicons are products of previous DNA amplifications that can contaminate the PCR process.

The master mix is the mixture of chemicals that must be added to the sample containing the target in a test tube before the PCR procedure begins. It is important to prepare this mix in a clean area away from products of previous amplifications called *amplicons*. Amplicons can also serve as templates in the PCR procedure and may provide false positive results. The master mix includes an excess of the four deoxynucleotide triphosphates (adenine, thymine, cytosine, and guanine); *Taq* polymerase; an excess of oligonucleotide primers; buffers; and magnesium chloride.

Thermal Cycler

The thermal cycler is a commercially available, programmable, microprocessor-regulated, heating and cooling block. This

instrument can be programmed for the temperatures and time required for each step in the PCR procedure. PCR can be time required for each step in the procedure and can also be performed in separate water baths for each temperature. This procedure is often not successful and takes much longer time to complete.

The thermal cycler regulates the temperature and length of time for each step in PCR.

During PCR, the sample of DNA being studied is added to the master mix that is placed into the test tube.

In a typical PCR procedure, the specimen to be amplified and the master mix are placed in a tiny, thin-walled test tube and overlaid with mineral oil to prevent evaporation. The tube is placed into the well of the thermal cycler, heated to about 90° C for one minute for dissociation, cooled rapidly to about 50° C for one minute to allow the primers to anneal, and then reheated to 72° C for one minute for the *Taq* polymerase to extend the primers. Forty cycles of this duration can be completed in two hours. Different procedures may have different optimal times and temperatures, depending on the length of the target, the length of the primers, and the predominant bases in the DNA.

Detection to Assure Amplification of the Right Sequence

Once the DNA sample has been amplified, it can be detected and identified using gel electrophoresis.

Once the DNA is amplified, it must be detected and identified. The conventional method is electrophoresis of amplification products in agarose or polyacrylamide gels (see Chapter 1). Once electrophoresed, the bands can be stained with ethidium bromide, a fluorescent compound that attaches to the DNA inside the gel. The bands produced can be compared with known positive and negative controls. These gels are then photographed with a Polaroid camera for documentation. A predominant, single band is seen in the gel following the basic PCR procedure described here.

Probes are single-stranded oligonucleotides that can attach to complimentary DNA.

PCR products can also be identified using DNA probes. These can identify the PCR products by hybridizing with the target. DNA probes are single-stranded oligonucleotides complementary to the target and are usually labeled for detection. The probe is immobilized on a solid support. This support can be a nylon membrane or a microtiter well. If complementary DNA is present in the PCR product, it will attach to the probes on the solid support. Unbound DNA is washed away. This hybrid is labeled with radioactive phosphorus, enzymes, or chemiluminescent acridium esters that produce a visible product. Radioactive phosphorous can then be detected by autoradiography. Enzymes can produce a color change visible in a spectrophotometer. Chemiluminescence can be measured with a luminometer.

Although a detailed explanation of PCR procedures is beyond the scope of this chapter, the following procedural description explains the basic steps in PCR.

Detailed PCR Procedure

1. Extract DNA from the nucleus of cells.
2. Prepare the master mix and add it to the extracted DNA in a test tube.
3. Place the test tube into a thermal cycler. Set the times and temperatures for each cycle and the number of cycles to be performed. When the procedure begins, there are long strands of DNA that may contain the target present in the test tube. If at least one target is present in the sample, the following steps will occur. If more than one target is present, then multiples of this number of targets will be produced. In other words, if 20 copies of the target DNA are present in the sample, multiply each *number of strands* in the example below by 20. Keep in mind that this is a theoretical maximum that is not achievable in actual practice.

CYCLE 1

One copy of double stranded DNA containing the target is present in the test tube (see Figure 15-3). The target sequence shown below is in bold type. The number of bases in this example is less than is optimal for demonstration purposes.



Figure 15-3

1. Heat to 95° C in the thermal cycler for one minute to denature the DNA. This results in single-stranded DNA (see Figure 15-4):



Figure 15-4

2. The thermal cycler cools the mixture to 55° C, and primers anneal to the long strands of DNA if the target is present in the sample. Each primer anneals to the 5' prime end of the strand that is complementary to it when the temperature drops (see Figure 15-5).



Figure 15-5

3. The thermal cycler heats the mixture in the test tube to 72° C. Then *Taq* DNA polymerase extends the primers by adding nucleotides to the primers that are complementary to the template strand. The polymerase continues to add bases along the entire long strand of DNA past the target site until the temperature is raised and the strands become single stranded again. At the end of this first cycle, there are four copies of each target present in the test tube: the two original long strands and two shorter midlength strands that begin at a primer site and extend to various lengths at the 3' end (see Figure 15-6).



Figure 15-6

CYCLE 2

In the second cycle, all four strands serve as templates for replication. Primers anneal to the two original long strands again and are extended from the primer site toward the 3' end past the target sequence, making midlength strands. Primers also anneal to the two midlength strands made in the first cycle. These two midlength strands are extended from the primer site to the shorter end of the strand making two short strands.

1. The thermal cycler heats to 95° C for the second cycle, and the strands come apart and become single-stranded (see Figure 15-7).

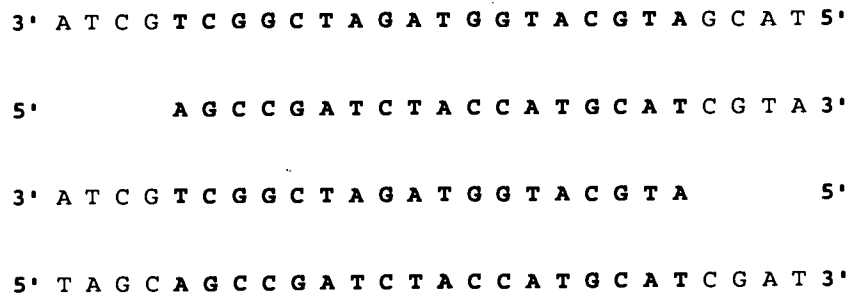


Figure 15-7

2. The thermal cycler cools to 55° C and the primers anneal to all four strands (see Figure 15-8).

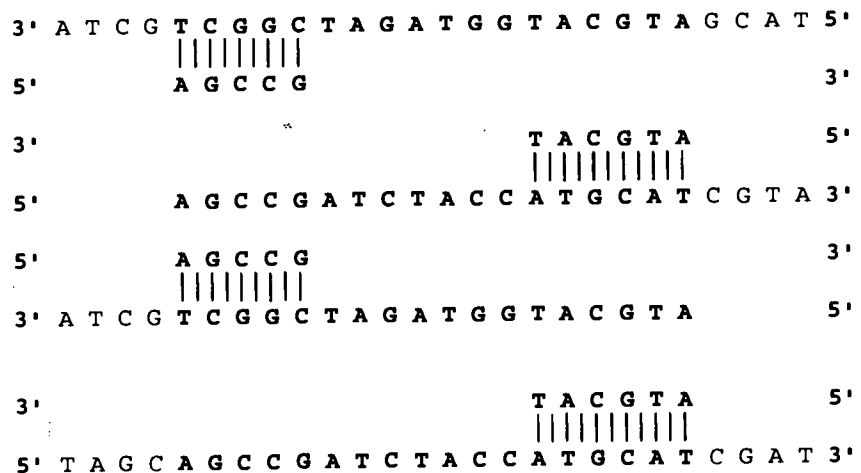


Figure 15-8

3. The thermal cycler heats to 72° C, and *Taq* polymerase extends the primers (see Figure 15-9).

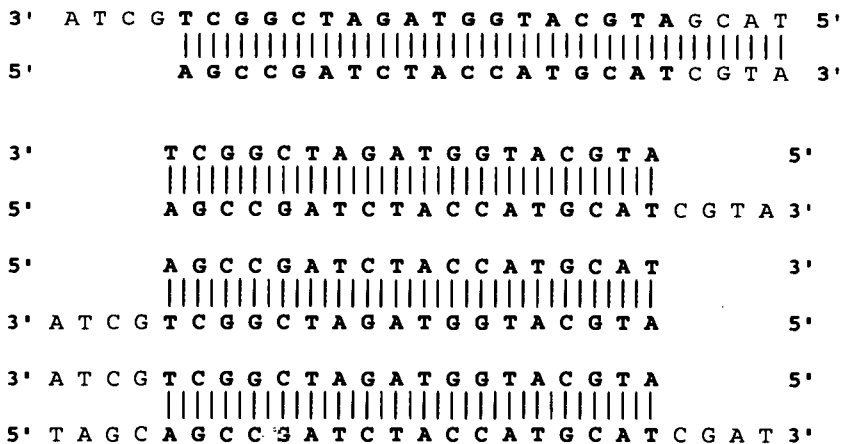


Figure 15-9

CYCLE 3

In the third cycle, all eight strands serve as templates. This time, duplicates are made of the two original long strands making two midlength strands. The four midlength strands, the two made in the first cycle and the two made in the second cycle, now are extended to make four short strands. The two short strands made in the second cycle are now extended to make two more short strands. This makes a total of 16 templates: two long, six midlength, and eight short.

1. The thermal cycler heats to 95° C for the third cycle and the strands come apart and all eight are single-stranded (see Figure 15-10).

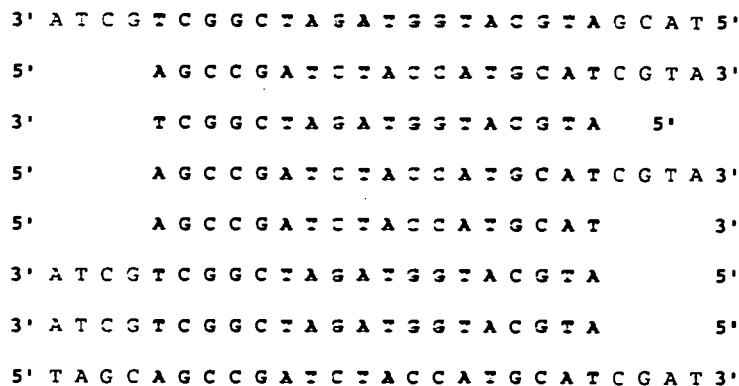


Figure 15-10

2. The thermal cycler cools to 55° C, and the primers anneal to all eight strands (see Figure 15-11).

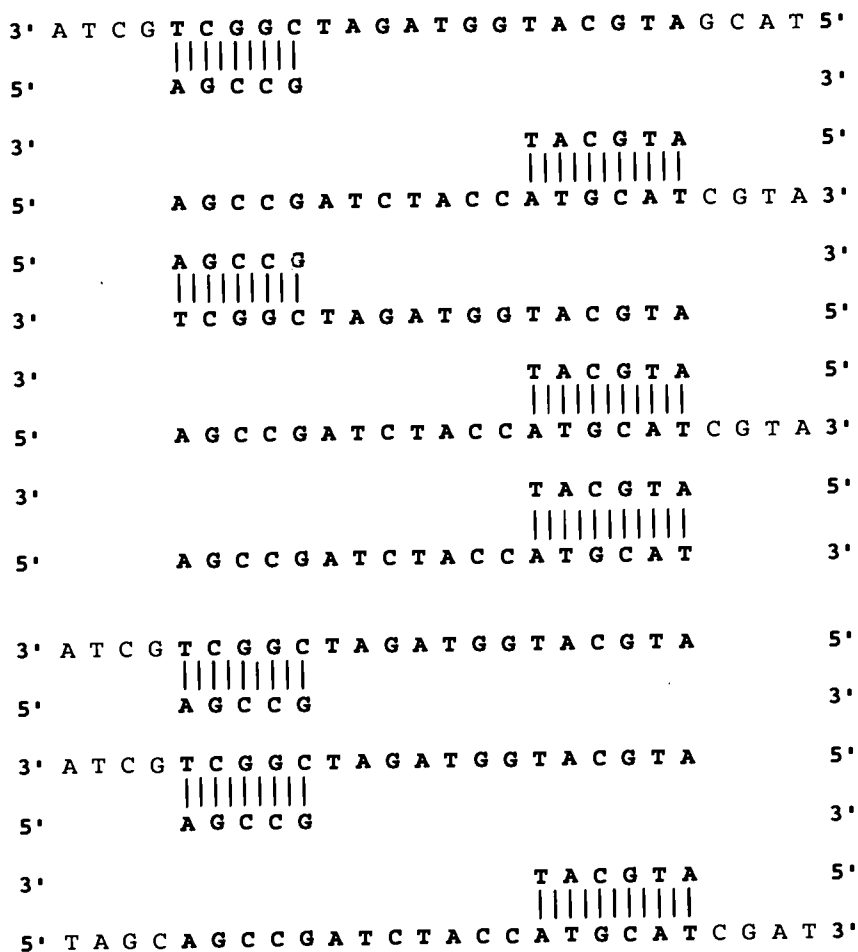


Figure 15-11

3. The thermal cycler heats to 72° C, and *Taq* polymerase extends the primers (see Figure 15-12).

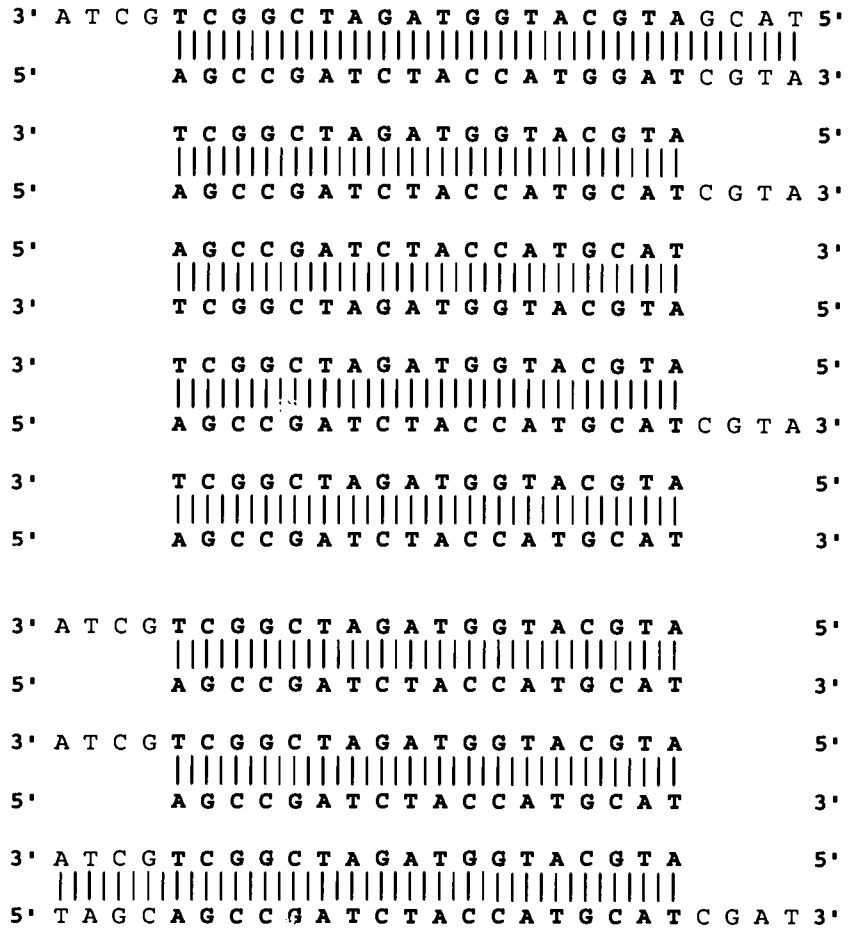


Figure 15-12

CYCLE 4

The fourth cycle continues in the above manner. The original long strands remain to make more midlength strands, but all the midlength strands are extended to make more short strands. The midlength strands increase arithmetically, but the short strands increase exponentially. At the end of the fourth cycle, there will be the original two long strands, eight midlength strands (two made from the long strands in each of the previous cycles), and 22 short strands: 14 new short strands (made from the eight short strands and the six midlength strands from Cycle 3) plus the eight short strands present at the end of Cycle 3. This makes a total of 32 strands, with most being short strands. As the cycles continue, more and more short strands will be produced, but only a few more midlength strands will be produced. Therefore, at the end of 30 cycles, there will be only a small number of midlength strands but more than a billion identical short strands defined by the primer pairs.

1. The thermal cycler heats to 95° C for the fourth cycle, and the 16 strands come apart and become single-stranded (see Figure 15-13).

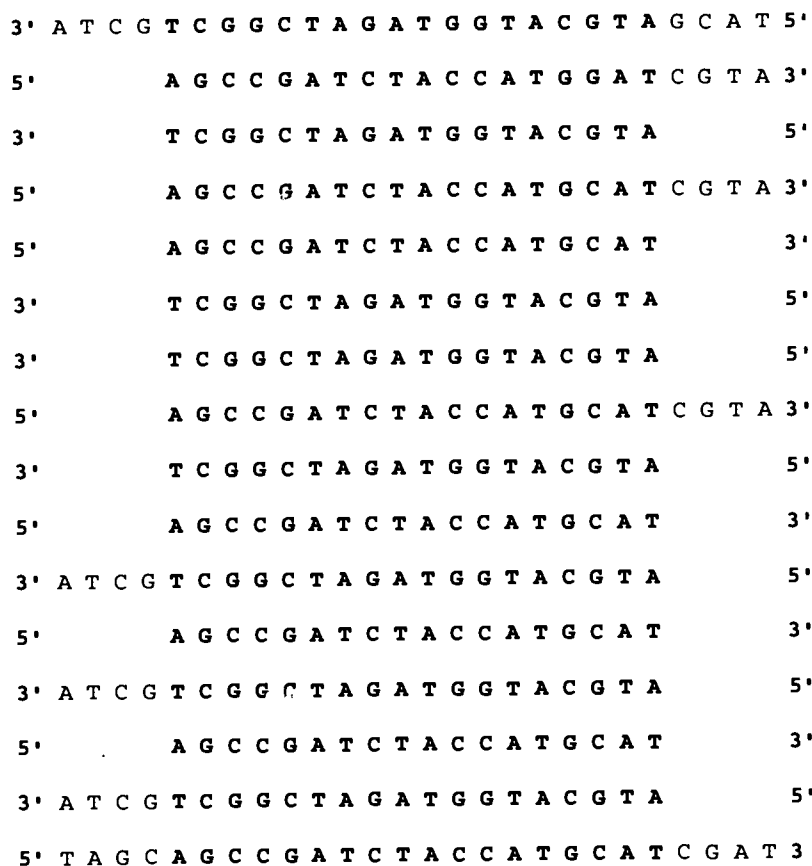


Figure 15-13

2. The thermal cycler cools to 55° C, and the primers anneal to all 16 strands (see Figure 15-14).

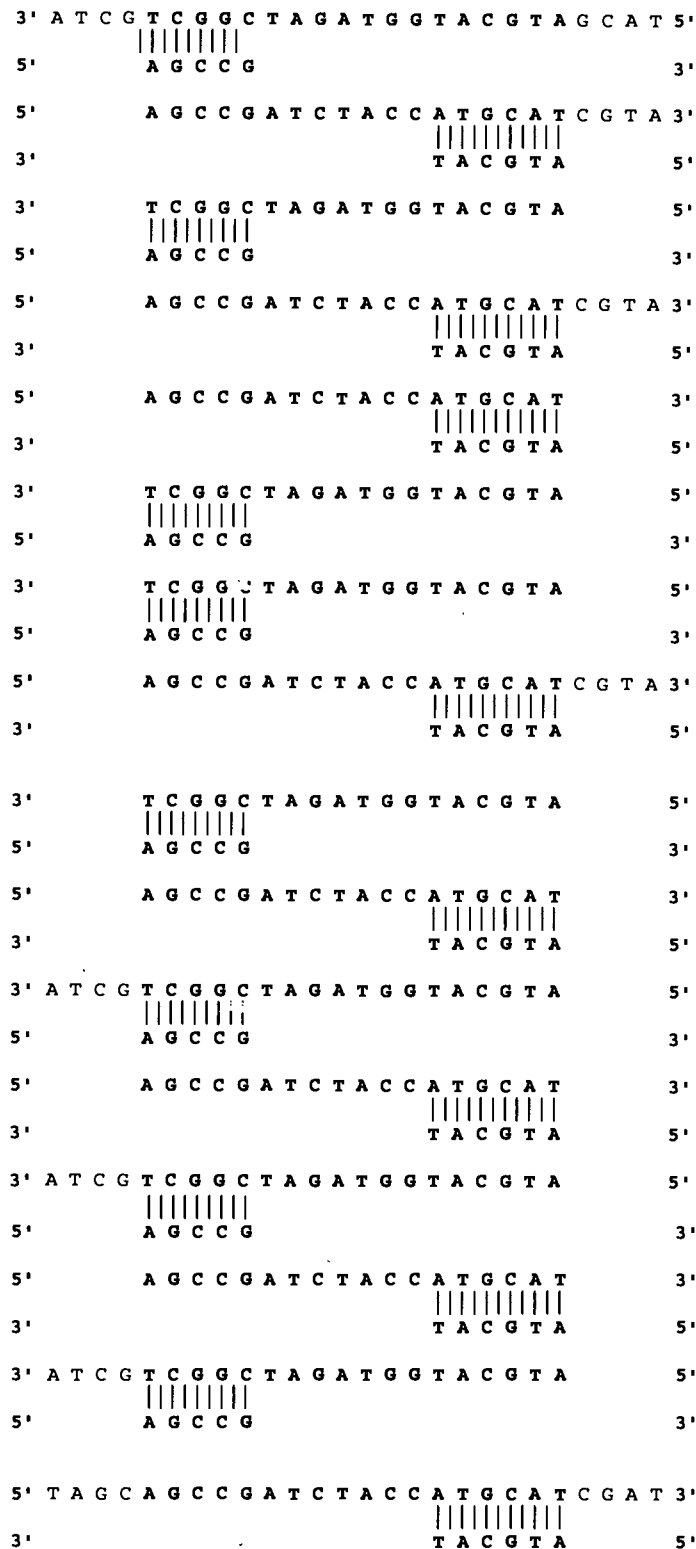


Figure 15-14

3. The thermal cycler heats to 72° C, and *Taq* polymerase extends the primers. There will be two original long strands, eight midlength strands, and 22 short strands for a total of 32 strands. You can work it out for yourself. The rule is: 2^{n+1} equals the total strands at the end of each cycle, where n is the number of cycles. The number of midlength strands will increase by two with each cycle. The equation for midlength strands is $2n$, where n is the number of cycles completed. The number of short length copies can be calculated by subtracting the number of original and midlength copies from the total at the end of the cycle.

Figures 15-15 and 15-16 are summary diagrams for PCR.

Applications of PCR

PCR is commonly used to identify a particular sequence of nucleic acids in a test sample to aid in the diagnosis of disease.

The most important purpose for performing PCR is to determine if a particular sequence of nucleic acid is present in a test sample. Selective amplification of a unique region of mammalian DNA to aid in the diagnosis of disease is the most common use.

PCR is used in genetic studies and in the Human Genome Project.

Genetic disease can be detected by identifying point mutations, deletions, and additions in a person's DNA. This was first performed for sickle-cell anemia by identifying the defect in the hemoglobin gene. PCR is routinely used to diagnose cystic fibrosis, fragile X syndrome, Huntington's disease, and heritable diseases in which a specific genetic alteration has been identified. Direct gene analysis has allowed physicians to make a prenatal diagnosis and to predict the onset of diseases prior to symptomology. Gene therapy has been facilitated by PCR through providing copies of genes that can be inserted into defective cells. The Human Genome Project has greatly benefited from PCR by saving time and money when sequencing unknown DNA. Sequences that flank known sequences can be mapped by amplifying these areas and then determining the sequence of the nucleotides. Eventually, a complete chromosome can be mapped this way.

PCR can aid in the diagnosis of cancer.

PCR aids in the detection of the genetic alterations of cancer. Cancers can be associated with a mutation, the absence of specific tumor suppressor genes, or the activation of certain cancer-causing oncogenes. These alterations in DNA can cause normal cells to convert to cancer cells. Such alterations can be identified more easily by making copies using PCR. Identifying

284 Polymerase Chain Reaction

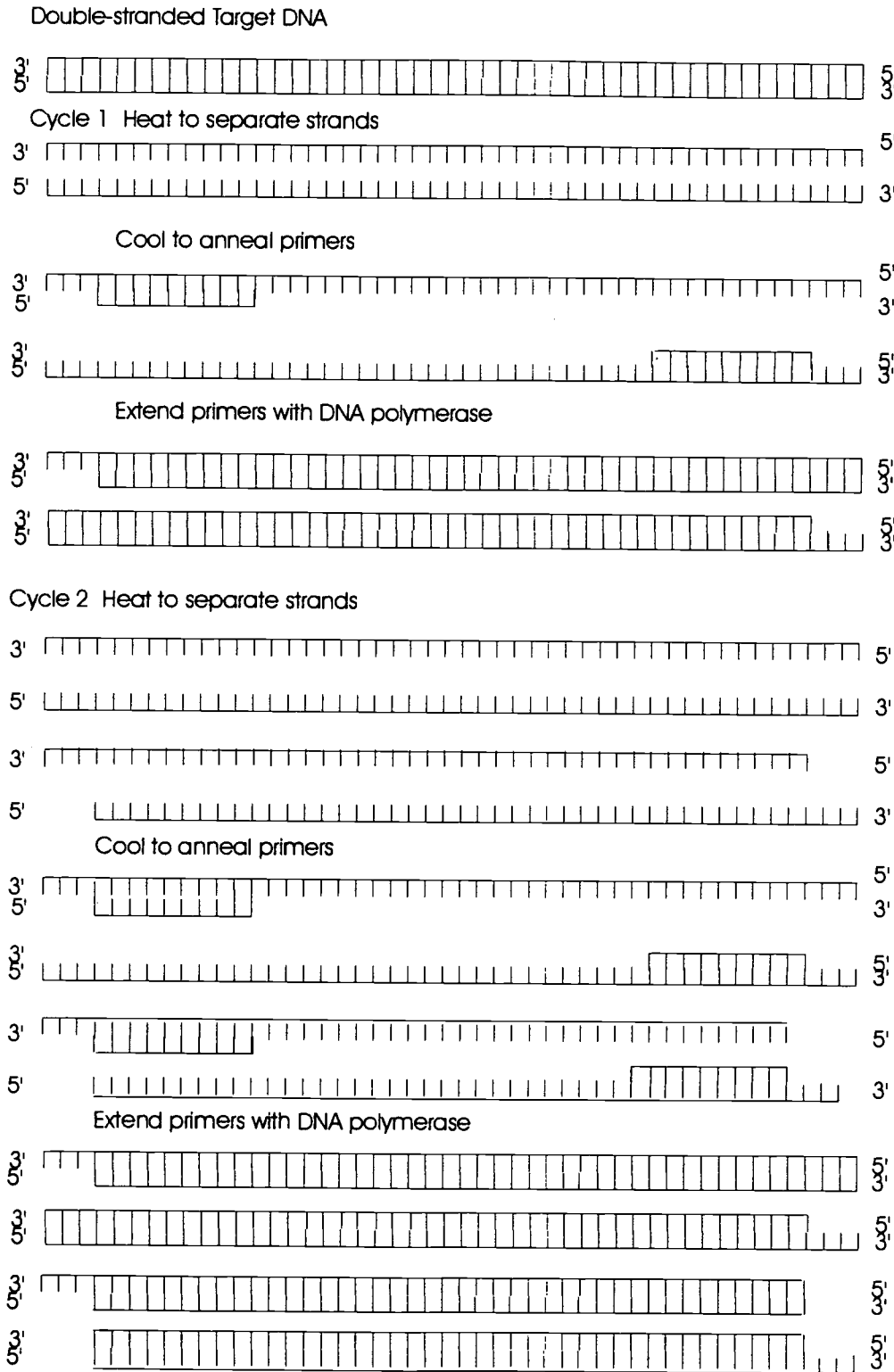
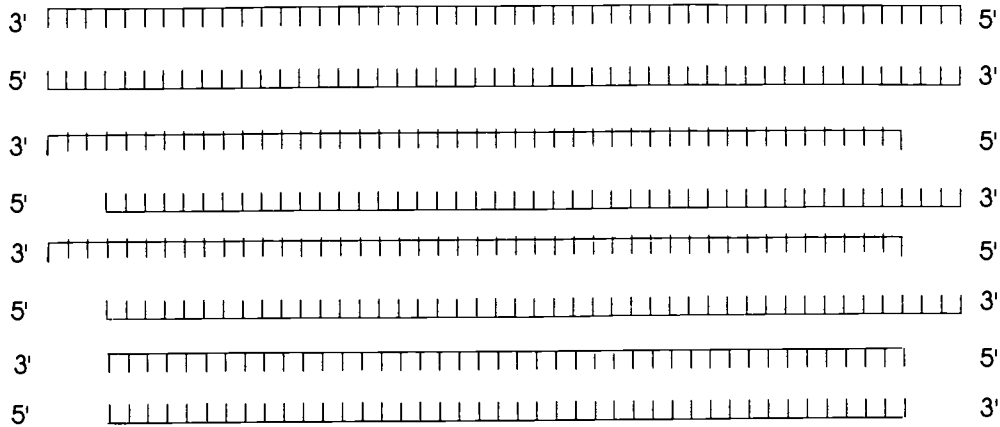
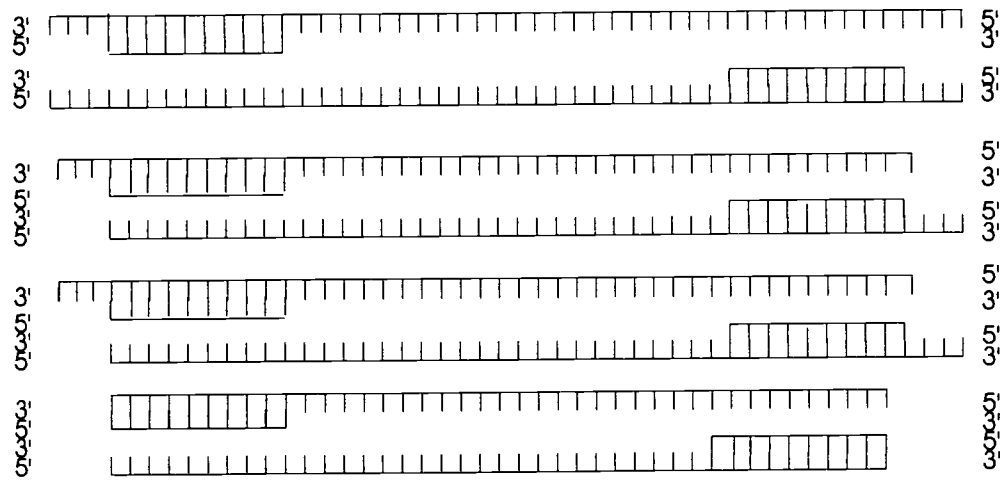


Figure 15-15
PCR part one.

Cycle 3 Heat to separate strands



Cool to anneal primers



Extend primers with DNA polymerase

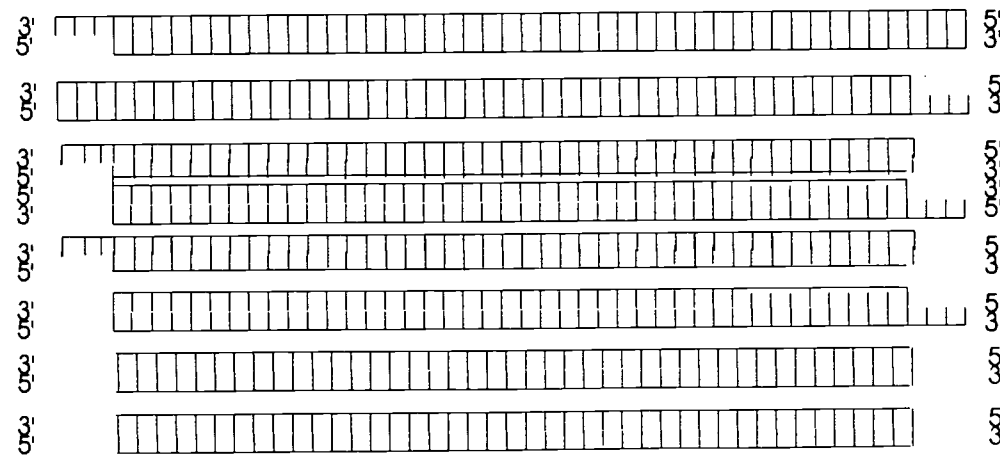


Figure 15-16
PCR part two.

individuals who have a genetic susceptibility to cancer via PCR can facilitate diagnosis of cancer cell type and guide early treatment.

PCR can assist in monitoring cancer treatment.

PCR can be used to monitor cancer treatment by detecting small numbers of cancer cells in large numbers of normal cells. This is particularly useful in bone marrow transplantation. A cancer patient's blood is collected, and stem cells are harvested and frozen. Stem cells are immature cells that develop into red blood cells, white blood cells, and platelets. The patient is given massive doses of chemotherapy that destroy bone marrow. After the chemotherapy has (hopefully) killed the cancer cells, the patient's stem cells are transfused back into his or her body to produce new bone marrow. The DNA of any cancer cells remaining in the stem cells can be detected prior to reinfusing the marrow, after amplification with PCR.

PCR can detect the presence of infectious microbes in various body fluids and tissues.

Infectious agents such as bacteria, viruses, fungi, and parasites can be detected by PCR. These agents can be present in blood, urine, sputum, tissue, water, and food. PCR is most useful when the infectious agent is difficult to culture, such as the human immunodeficiency virus (HIV) and the bacterium that causes Lyme disease. Some disease-causing organisms are unculturable but may be detected in tissue samples after amplification. Whipple's disease and Cat Scratch Fever are two examples. Hepatitis virus cannot currently be cultured, but its DNA can be amplified from blood samples. PCR can also be used to quantify the amount of target present in order to monitor antimicrobial therapy.

PCR is widely used in forensic medicine.

PCR has also been shown to be invaluable in forensic work. Unknown human and animal remains can be identified by comparing their DNA to that of a relative (or stored samples of their own DNA). The armed forces are now storing blood samples from all enlisted men and women so that casualties can be identified. Remains found in Vietnam have been identified by extracting DNA from an individual's bones, amplifying it, and comparing it with the relatives of the soldiers missing in action.

PCR can also be used to amplify DNA from blood, semen, and hair found at crime scenes, by comparing it with the suspect's DNA. Innocent men have been released from prison when the PCR analysis of crime scene evidence did not match their DNA. Paternity testing and determination of familial relationships can be carried out by amplifying and analyzing the HLA-DQ genes.

Maternal relatedness can be detected by examining mitochondrial DNA, which is only inherited from the mother.

PCR is used in the study of human evolution.

Mitochondrial DNA analysis has also been useful in establishing evolutionary relationships. This analysis currently supports the hypothesis for the African origin for human mitochondrial DNA. PCR can amplify the DNA for phylogenetic studies, allowing scientists to compare the DNA of contemporary species with that of extinct species. DNA from mummies has been amplified, and infectious agents, such as tuberculosis bacterium, have been identified as probably infecting the mummified human. Insects trapped in amber millions of years ago have been analyzed through PCR.

PCR can be used in ecological and population studies.

PCR is also invaluable to conservationists. It facilitates the identification of body parts belonging to protected species, such as elephant ivory collected illegally. Population genetics of species in decline can be studied more easily with PCR. Scientists can use PCR to monitor genetically engineered microorganisms in the environment. Even small numbers of pathogens can be identified in drinking water, for example, indicating fecal contamination. *Legionella*, the cause of Legionnaire's disease, a type of pneumonia, has been detected in water from hot tubs or air conditioning units by PCR.

Other Methods for Amplification of DNA or RNA

PCR is not the only procedure for amplifying DNA. There are many modifications of PCR. Nested PCR, multiplex PCR, PCR amplification of RNA, *in situ* PCR, quantitative PCR, and capture PCR are just some of the emerging technical modifications. There are two general strategies for amplification: systems that produce new copies of the original target nucleic acids which include the polymerase chain reaction and self-sustained sequence replication (3SR) and probe amplification systems which include the ligase chain reaction (LCR) and Q-beta replicase (QBR).

Advantages and Disadvantages of PCR

The greatest advantage of PCR, its exquisite sensitivity, is also its greatest drawback. Contamination from carryover between samples and from previous amplifications must be stringently monitored. There are different methods for preventing

contamination, and each laboratory must pursue one or more of these to prevent false positive reactions. Reaction inhibitors in the specimens, such as detergents, proteases, and chemotherapeutic agents, can cause problems. Dilution or chelation of the sample may prevent inhibition of amplification.

Despite its disadvantages, PCR remains the most widely used procedure for increasing small amounts of impure DNA to detectable levels. It is straightforward and versatile. It can be performed easily in the laboratory with the right equipment and close attention to detail. PCR is presently the most powerful tool for the detection of specific nucleic acid sequences, and it can replace many conventional hybridization assays.

Commonly Asked Questions by Students

Question 1: How do the primers know where to attach?

The primers are complementary to the bases in the target sequence. If the target contains adenine, only thymine or uracil will pair with it. If the target contains guanine, only cytosine will pair with it. The primers are chosen to pair only with the particular sequences that are on each end of the target. They can only pair with that part of the DNA.

Question 2: Why does only the target become amplified?

The primers determine the specificity. They must be unique to the target sequence. If they are unique, and the conditions of the test are met, they will bracket only the target sequence. By duplicating only the DNA between two primers, there will be exponential growth of the target sequence.

Question 3: What happens if previously amplified DNA is present in the solution or master mix?

Unless a special enzyme is added to prevent the attachment of previously amplified DNA, it will be amplified along with any new target in the test. This contamination can result in a false positive test or other confounding problems.



Lynda Britton, instructs Stephanie Mason, senior medical technology student at LSU Medical Center, School of Allied Health Professions in Shreveport, LA, on how to perform PCR using a thermal cycler.

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About the Author

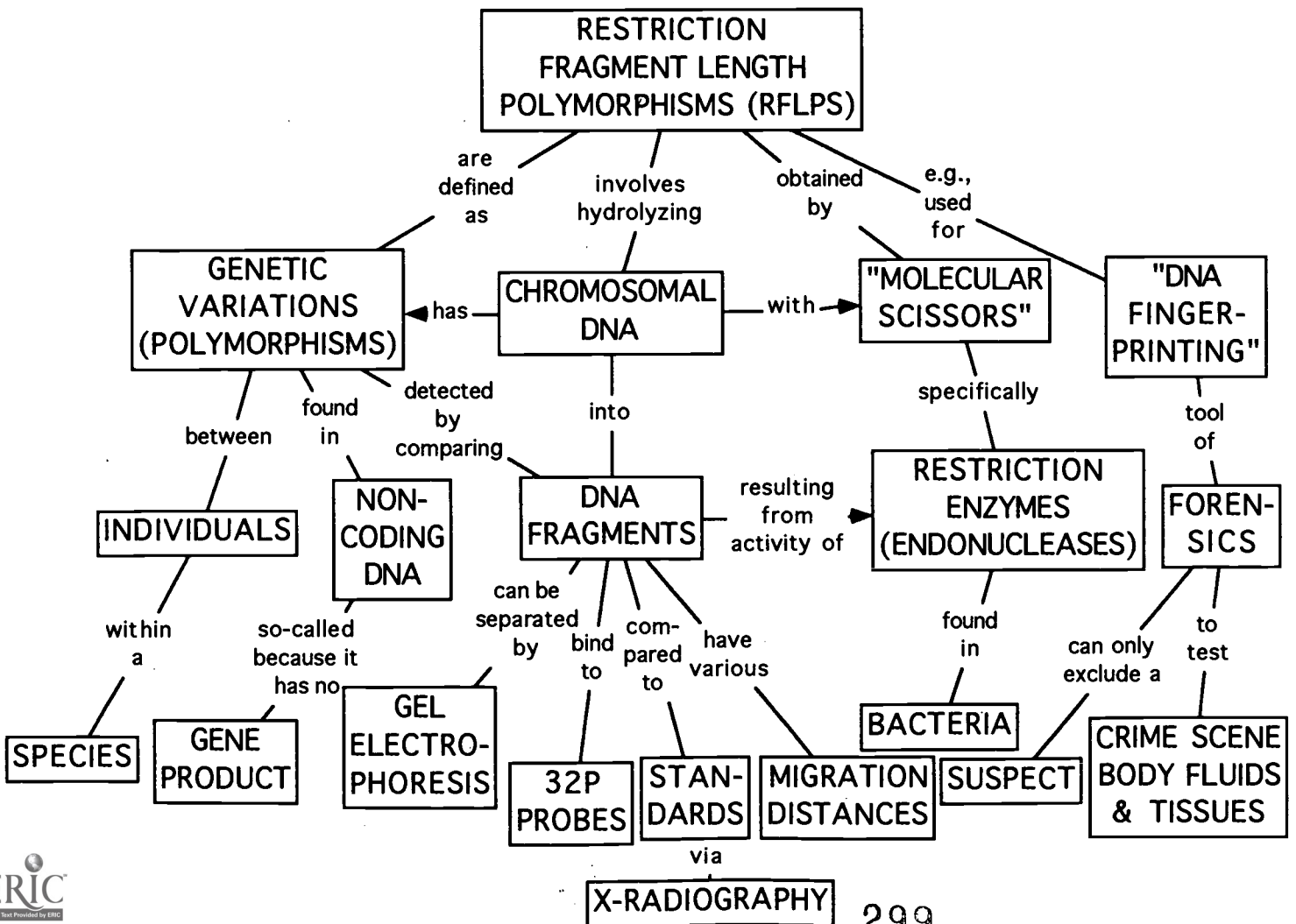
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RESTRICTION FRAGMENT LENGTH POLYMORPHISMS (RFLPS)

16

Janice Matthews-Greer

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1. Review of genetics
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Restriction Fragment Length Polymorphisms (RFLPs)

Definition

The term, *Restriction Fragment Length Polymorphisms*, in its most proper abbreviated form, is *RFLPs*. Many authors prefer the ease of pronunciation of “riflips” or “reflips.” All three are found in the literature. RFLPs are broadly defined as genetic variations (polymorphisms) between individuals, detected by comparing the fragments (size and/or number) resulting from cutting their chromosomes with special enzymes (restriction endonucleases). Other terms used in connection with RFLPs include *DNA fingerprinting* and *DNA typing*.

RFLPs are genetic variations between individuals detected by cutting chromosomes with special enzymes.

First: A Quick Review of Genetics

A brief review of genetics will assist the reader in understanding RFLPs. *Mitosis* is the type of cell division resulting in growth. The two resulting daughter cells are essentially “carbon copies” of the parent cell. In contrast, *meiosis* is referred to as reduction-division because, after two rounds of division (meiosis I and meiosis II), four daughter cells are formed, each with half the chromosome content of the original parent cell. During meiosis, a diploid cell (one in which the chromosomes exist in pairs) produces four haploid cells (cells with only one member of each pair of chromosomes). In higher organisms, mitosis occurs in somatic (body) cells, and meiosis occurs in cells found in the reproductive organs (e.g., flowers, testes, or ovaries).

Diploid organisms (such as humans) have chromosomes that exist as pairs of homologous (similar) chromosomes in their somatic cells. In contrast, only one of each kind of chromosome is found in haploid gametes (eggs and sperm). When fertilization takes place, diploidy is restored, with one member of each resulting homologous pair being of maternal origin (from the egg) and the other member being of paternal origin (from the sperm). Therefore, in each human body cell, there are 23 pairs of chromosomes or, to be more precise, 22 pairs of autosomes and one pair of sex chromosomes.

A gene is a section of a chromosome — a unit of heredity. A gene is often thought of as being a region of DNA that encodes for a protein or for RNA. However, not all of the chromosome encodes for a “gene” product. Yet, even noncoding regions are inherited; they are still a part of the chromosome just

like any other “gene.” Some parts of the chromosome are invariant from individual to individual; whereas, other genes do vary. Genetic variations are called *alleles*. [For example, let us assume that on Chromosome #5 there exists a particular gene at a particular locus (location). On the other member of that homologous pair (the other Chromosome #5), the same kind of gene would be found at the same locus or site. These two genes may be exactly alike in their DNA sequence or they may be different alleles.] Some regions of the chromosome exist in different forms (different alleles); some do not; and some are highly variable (polymorphic). These ideas will be expanded below.

Allelic Variation (Polymorphism)

Polymorphisms are variations in DNA between individuals.

Polymorphism can be described as variations in DNA between individuals of a species. There are several million detectable differences in the DNA between any two individuals. We most often think of these differences as those that affect phenotypes (our physical traits). For example, polymorphism exists at the ABO blood type locus (site on a chromosome) for the gene for blood type in humans. To simplify, the A type, the B type, and the O type alleles are simple variations of the ABO blood type gene. When discussing a diploid individual, such as a human, it must be remembered that chromosomes exist in pairs. Therefore, our analysis must take into consideration both pair members of the gene of interest. An individual is heterozygous if she or he has two different alleles for a particular gene (such as B/O) and homozygous if the two alleles are the same (such as O/O). No matter how polymorphic the gene, any individual can only have two variations.

Many sequences in the human genome have no known coding function.

The smallest human chromosome contains 50,000 kilobases (kb) of DNA. (This translates into 50,000,000 base pairs. A kilobase is 1,000 bases, and the “pairs” is implied because DNA is a double-stranded nucleic acid.) The human genome is probably comprised of close to three billion base pairs. Many sequences in the human genome have no known coding functions — possibly most of our DNA. These sequences are not subject to the same selective pressures as those genes that encode for expressed proteins because there will be no gene product to be *selected for* or *against*. Thus, a high degree of polymorphism is tolerable in these regions. Indeed, one in every 200 to 500 nucleotides in noncoding regions is polymorphic. (Keep

in mind that even though they are in noncoding regions, these polymorphisms are passed on to the offspring as part of the chromosome, just like any other “gene” on the chromosome.)

Most eukaryotic genes have their coding sequences (exons) interrupted by noncoding sequences called introns.

Noncoding segments of DNA serve multiple functions such as regulating signals and structural blocks.

Restriction enzymes are used to cut chromosomal DNA

The location on DNA that is cut is determined by sequences referred to as palindromes.

Some of the noncoding segments of DNA are referred to as *introns*. An intron is an interruption in a coding gene, like ... *ah, well, you know...*, those terms used to interrupt everyday speech. Before the gene is expressed (before the protein is made), the information is rewritten into RNA, and the intron sequences are removed. Some of the noncoding regions serve as regulatory signals used to indicate the beginning or the end of a coding region. Some serve as structural blocks required for the proper physical function of the chromosomes, and some are still a mystery.

Restriction Enzymes

Central to the theme of RFLPs are the molecular scissors (*restriction enzymes* or *restriction endonucleases*) used to cut chromosomal DNA. Restriction enzymes are produced by bacteria for the purpose of hydrolyzing (cutting) DNA in specific locations. The exact location of the cut is determined by areas of two-fold symmetry called *palindromes*. (You may remember that a palindrome is a word that reads the same forward or backward; e.g., *radar, noon, sees, deed, mom, dad, eye, rotor*.) This reference to a palindrome, however, takes on a literary license in its adaptation to DNA because DNA is double-stranded.

The palindromic sequence recognized by the restriction endonuclease varies with the particular enzyme. For example, shown in Figures 16-1 and 16-2 are the cutting and recognition patterns for two restriction enzymes commonly used as tools in molecular genetics. The first is called Eco RI because it is purified from the bacterium, *Escherichia coli* (*E. coli*). This endonuclease recognizes the palindromic sequence shown in Figure 16-2A and will cut the DNA everywhere it “sees” this 6-base sequence. Note that the sequence GAATTC reads forward on one strand the same as it reads backward on the opposite strand. Eco RI cuts between the G and the A on both strands as shown in Figure 16-2B. (After the cuts are made, the G on the left top strand of Figure 16-2B will still be hydrogen-bonded to the C on the bottom left strand. Likewise, the C on the top right strand will still be hydrogen-bonded to the G on the bottom right strand.)

Figure 16-1A. The Eco RI recognition site.

```
..... GAATTC .....  
..... CTTAAG .....
```

Figure 16-1B. Eco RI cuts between the A and the G on both strands.

```
..... G   AATTC  
..... CTTAA   G.....
```

Figure 16-2A. The Hind III recognition site.

```
..... AAGCTT .....  
..... TTCGAA
```

Figure 16-2B. Hind III cuts between the two A's on both strands.

```
..... A   AGCTT  
..... TTCGA   A.....
```

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Two common restriction enzymes are:

- 1) *Eco RI*
- 2) *Hind III*

A different sequence is recognized by *Hind III*, a restriction enzyme purified from *Haemophilus influenzae*. *Hind III* recognizes the sequence shown in Figure 16-2A and produces a cut between the double A's as depicted in Figure 6-3B. In each of these examples (Figures 16-2 and 16-3), the sequence recognized by the enzymes (called a *restriction site*) comprises a six-base sequence. Also, both enzymes cleave the DNA strands at different points to produce staggered ends. This is not always the case. Some enzymes recognize a four-base sequence, and some enzymes cut the two strands at the same place to produce blunt-ended fragments.

The number of DNA fragments produced by a restriction enzyme depends upon the number of restriction sites.

When a piece of DNA is digested with an endonuclease, such as *Hind III*, the enzyme will cut the DNA at every restriction site. The number of fragments produced will depend on the number of restriction sites available on the DNA. Consider a linear piece of DNA that is 27 kb long. (This translates into 27,000 pairs of nucleotides.) In this piece of DNA, depicted in Figure 16-3A, the base pairs are represented as dashes; the arrowheads indicate the restriction sites for *Hind III*. The recognition sequence AAGCTT is found at each of the four arrowhead locations. Both strands would be cut.

Thus, the enzyme would cut the DNA into five fragments of different lengths. (These fragments are numbered in the order they appear on the length of the DNA.) The lengths of these five individual fragments would all add up to the total length of the undigested piece of DNA; that is, 27 kilobases.

RFLPs

Electrophoresis can delineate the number and length of DNA fragments

The number and lengths of these five DNA fragments can be delineated using a technique known as *agarose gel electrophoresis* (see Chapter 1). This technique separates particles by their migration distances through an electrical field. Agarose is a seaweed extract with the texture of porous Jell-O[®]. These pores allow the particles to separate according to their size. (Larger particles are retarded in their movement, whereas smaller particles pass easily [and faster] through the pores.) Sample size is determined by comparing the distance traveled by a fragment to the distance traveled by DNA standards of known size. After electrophoresing the digested DNA from Figure 16-3A, the gel might look like the diagram shown in Figure 16-3B. In this representation, the bands (seen as dashed lines) are DNA fragments. The molecular weight standard is in Lane

Figure 16-3A. A DNA Sequence, 27 kb Long

The dashes represent base pairs. The arrowheads represent the Hind III recognition site, and the numbers are the fragments that would result from a Hind III digestion.

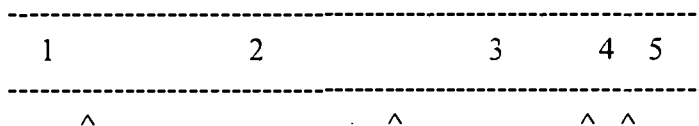
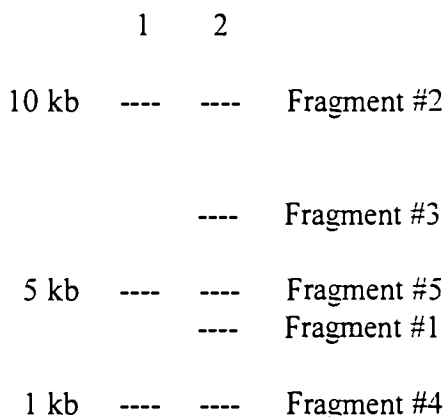


Figure 16-3B. Diagram of an agarose gel of the digested fragments from Figure 3A.

The dashed lines represent the bands of DNA created by the electrophoretic separation of DNA fragments. In Lane 1 is a standard comprised of DNA pieces of a known size. This standard is used as a ruler to measure the sizes of the unknown fragments in Lane 2. Lane 2 contains the fragments from the digested DNA from Figure 16-3A.



1 (column 1). The standard consists of DNA fragments of various sizes as labeled (10 kb, 5 kb, and 1 kb). Note that the direction of electrophoresis (direction of migration) is from the top of the paper downward. In Lane 2 are the digested DNA fragments labeled according to their location in Figure 16-3A.

In this fictitious example, Fragment #1 measures 4 kb; Fragment #2, 10 kb; Fragment #3 is 7 kb; Fragment #4, only 1 kb; and Fragment #5 is 5 kb. (These add up to 27 kb). Note that the fragments migrated according to their size, not their location in the original piece of DNA. Fragment #2 was the largest and migrated the slowest. Fragment #4 was only 1 kb in size, and it "ran" the fastest.

Assume that in the original piece of DNA (from Figure 16-3A) a mutation occurred. This mutation in the last recognition site (at the last arrowhead) changed the first A of the recognition sequence to a G (AAGCTT became GAGCTT). If that happened, then the enzyme would only cut the fragment at three locations to produce only four fragments. The fragment sizes still would be #1 with 4 kb, #2 with 10 kb, and #3 with 7 kb. However, the 1-kb and 5-kb fragments would be missing. In their place would be a 6-kb fragment. Such a situation is shown in Figure 16-4A. The resulting agarose gel would look like Lane 3 in Figure 16-4B. (Compare Lanes 3 and 2. Note the differences indicated by the starred bands.) Lanes 1 and 2 are just like those of Figure 16-3B.

In the example in Figure 16-4A, the substitution of a G for an A on the DNA created a *restriction fragment length polymorphism*. That is because the resulting allele or variant with the mutation (shown in Lane 3 of Figure 16-5B) has a different restriction fragment migration pattern from that of the original allele (in Lane 2). Therefore, Hind III digestion was used to distinguish between these two alleles by RFLP analysis. It can be summarized by stating that the patterns of cutting by the enzyme vary between these two individuals because there are differences in the DNA sequences that can be detected as RFLPs. The sequence of DNA represented by the original DNA (from Lane 2) and the sequence of DNA represented by the mutated DNA (from Lane 3) would be *heritable*. Just like the blue and brown alleles for the eye color gene, each of the two variations seen as RFLPs could be considered an allele passed on to progeny cell generations. If a parent had one chromosome with the mutation, then there is a 50% chance

The difference between individual DNA molecules results in different patterns cut by the enzyme.

Figure 16-4A. The DNA sequence with a substitution mutation.

The DNA sequence shown is just like that of Figure 16-3A, but the restriction site between Fragments #4 and #5 no longer exists. Therefore, no cut would result at that location.

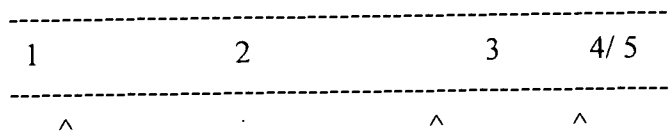


Figure 16-4B. Diagram of an agarose gel from the DNA sequence with a substitution from Figure 16-4A.

Lanes 1 and 2 are just like Figure 16-3B. Lane 3 contains fragments from the digest of Figure 16-4A. Note the RFLPs that are starred.

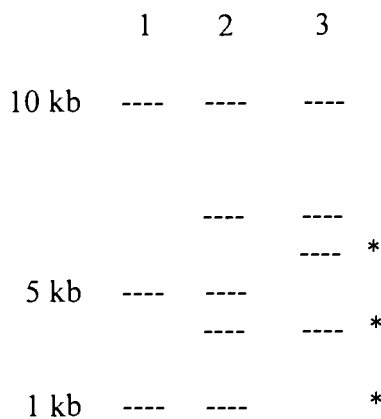


Figure 16-5A. The DNA sequence with an insertion.

The DNA sequence shown is just like that of Figure 16-3A, but 500 bases (0.5 kb) have been inserted at the site indicated by the + signs.

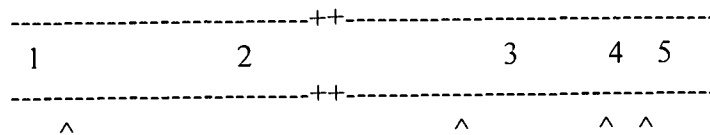
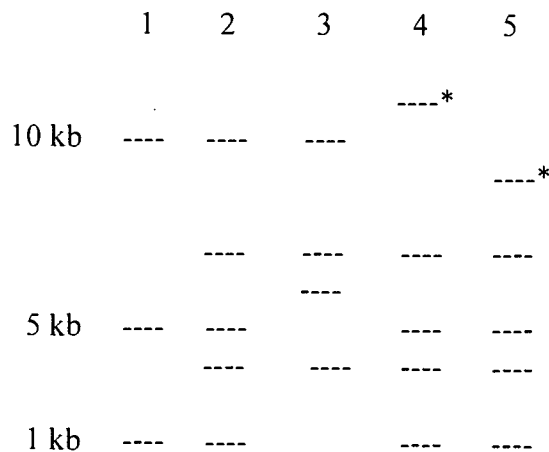


Figure 16-5B. Diagram of an agarose gel from the DNA sequences with an insertion and a deletion from Figure 16-5A.

Lanes 1, 2 and 3 are just like Figure 16- 5B. Lane 4 contains digested fragments from Figure 16-5A. The 500 base insertion has increased the size of 1 of the fragments. In e 5, one can see the results of a 500 base deletion from the original DNA strand (from Figure 16-3A). The RFLPs result-ing from both the insertion and the deletion are represented by stars.



that an offspring would inherit that mutation. If a parent had the mutation on both chromosomes, then the offspring would have a 100% chance of inheriting that mutation.

Suppose that on the original piece of DNA (Figure 16-3A) a mutation occurred, but not at a restriction site. Instead, a mutation occurred that inserted several additional bases (e.g., 500 base pairs) into the site on Figure 16-5A indicated by the + signs. How might this change the pattern of the resulting Hind III digested fragments? Look at Lane 4 in Figure 16-5B. Assume that Lanes 1-3 are the same as in the previous diagrams. In this new situation, the longest fragment has grown from the original 10 kb size to its new size of 10.5 kb. Likewise, if the DNA had a deletion of 500 bases, the 10 kb fragment would be reduced to 9.5 kb. (See Lane 5 of Figure 16-5B.)

Detection of RFLPs with Probes

To understand how RFLPs are detected, it is necessary to consider the methods associated with RFLP analysis. For example, because of the huge amount of DNA found in cells (three billion base pairs in humans — not just 27 kilobases), enzyme digestion would yield a thick smear on a stained gel. Therefore, there is a need for a detection system to visualize the band patterns of interest or some way to identify them within the huge mass of digested DNA fragments. For this purpose, probes are used in a process known as a *Southern blot*. Probes are polynucleotides that range in length from 20 to thousands of bases long. They are *complementary* to (bind to) part of the chromosome, in particular a “gene” segment found on the fragments that vary in length. This is described more fully in the next paragraph.

Polynucleotide probes are used to identify band patterns on the stained gel.

Complementary refers to the ability of the probe to bind to the chromosome.

In Southern blotting DNA is denatured prior to transferring fragments on to the membrane.

Before the probe can actually hybridize (bind by hydrogen bonds) to the target DNA, the target fragments must be transferred from the gel onto a paper or nylon membrane. They also must be denatured (i.e., made single-stranded.) Remember that cellular DNA consists of two long polynucleotide strands that are hydrogen bonded together to form the double-stranded molecule. The cytosine bases on one strand are complementary to, and thus hydrogen can bond to, the guanine bases on the opposite strand.

Likewise, the adenines of one strand are bound to the thymine bases on the opposite strand. Therefore, before the

probe can hydrogen bond to its complementary target sequences (C to G and A to T), the target must be made single-stranded by breaking the hydrogen bonds that hold the two strands together. Then the bases are free to hydrogen bond to the probe *if* the sequence is complementary. (A probe with the sequence AAAGG would hybridize to a target containing TTTCC.) Either heat or a strong base solution can break hydrogen bonds and thus denature the DNA on the gel. Complementarity is depicted in Figure 16-6. This diagram of a “miniaturized” probe shows how a probe might bind to its complementary region. In Southern blotting, the gel is usually submersed in alkali (sodium hydroxide) to denature the DNA before transferring the fragments to the membrane. This will be discussed in more detail in the methods section.

In the example from Figure 16-5B, a scientist might choose to use two probes, one (logically named P1) which is complementary to the 10-kb fragment, and one (P2, of course) which is complementary to the 1-kb fragment from the original strand of DNA (see Figure 16-3A) to use as probes to react with the four different variations of DNA. These probe-binding regions are shown by the “P1” and “P2” notations on Figure 16-7A. Such probes are added to the membrane containing the digested fragments to see where they bind. This indicates which fragments are similar in *sequence*, if not in size.

An example of probe binding to DNA fragments is depicted in Figures 16-7B and 16-7C. First, using the P1 probe that is complementary to the 10-kb fragment of the original DNA, a pattern shown in Figure 16-7B would develop. Recall that Lane 1 in Figure 16-7B is the molecular weight standard. It is unrelated DNA (often bacteriophage DNA is used), so it would not bind to the DNA probes. Lane 2 is the digested original DNA (from which the probe was made). Lane 3 is the digested DNA with the substitution mutation. Lane 4 contains the digested DNA with the 500-base insert, and Lane 5 contains the digested DNA with the 500-base deletion.

The pattern formed by adding the P2 probe (complementary to the 1-kb fragment of the original DNA) would be as shown in Figure 16-7C. Why was the 6-kb fragment in Lane 3 detected with the P2 probe if the probe was complementary to the 1-kb fragment of the original DNA? Remember that the di-

Figure 16-6. An example of probe-binding to a segment of DNA by means of complementarity. Double-stranded DNA is denatured into single strands. *When mixed with the DNA strands, the molecular probe (shown with the sequence TTGCTAGGC) will bind to its complementary region. This binding is due to conventional hydrogen bonding between the nucleotide bases (A to T and G to C).*

Double-Stranded DNA	-----> denatured	Single Strands of DNA	-----> Add the Probe (TTGCTAGGC)	Probe Binding	
C - G		C	G	C	G
C - G		C	G	C	G
A - T		A	T	A	T
A - T		A	T	A	T
T - A		T	A	T	A
G - C		G	C	G	C
T - A		T	A	T	A
A - T		A	T	A	T
A - T		A	T	A	T
T - A		T	A	T	A - T
T - A		T	A	T	A - T
G - C		G	C	G	C - G
C - G		C	G	C	G - C
T - A		T	A	T	A - T
A - T		A	T	A	T - A
G - C		G	C	G	C - G
G - C		G	C	G	C - G
C - G		C	G	C	G - C
T - A		T	A	T	A
T - A		T	A	T	A
A - T		A	T	A	T
A - T		A	T	A	T
A - T		A	T	A	T

Figure 16-7A. The original DNA sequence with the probes bound. The P1 probe complementary to a portion of the 10-kb fragment of the original DNA strand (Fragment #2). The P2 probe is complementary to a portion of the 1-kb fragment of the original DNA strand (Fragment #4).

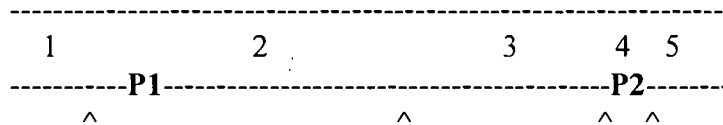


Figure 16-7B. Southern blot with probe P1 binding to electrophoretically separated DNA fragments from Figure 16-5B.

All of the Lanes contain the same samples as in Figure 16-5B. But, instead of staining the gel, the fragments have been transferred to a nylon membrane and reacted with probe P1. None of the fragments in the molecular weight standard in Lane 1 reacted. Note that the RFLPs were detected using the P1 probe—Lanes 4 and 5 differ from Lanes 2 and 3, and they differ from each other.

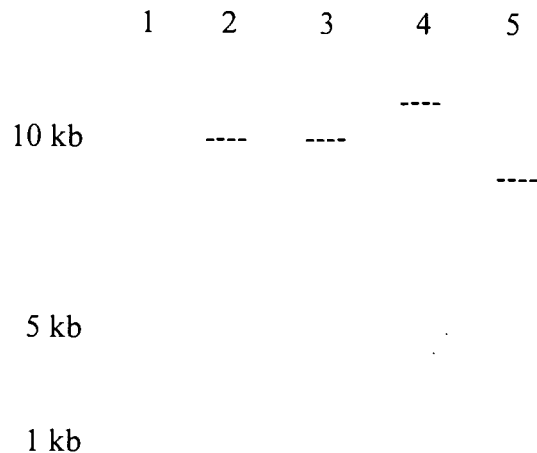
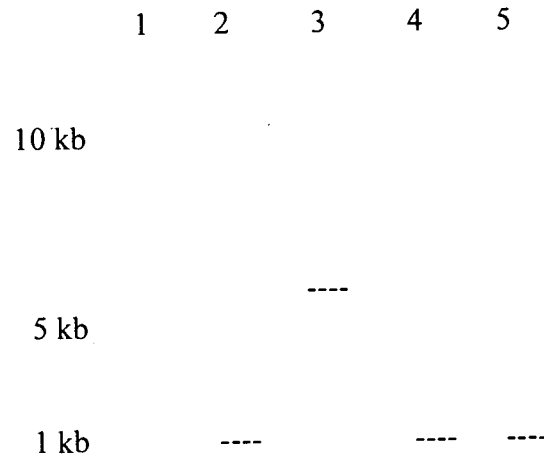


Figure 16-7C. Southern blot of probe P2 binding to electrophoretically separated DNA fragments from Figure 16-5B.

The lanes are as described in Figure 16-5B; but, instead of staining the gel, the fragments were transferred to a nylon membrane and reacted with probe P2. Again, none of the fragments from the molecular weight standard reacted. Note that a RFLP is evident—Lane 3 differs from the other pattern.



gested DNA in Lane 3 contained a substitution mutation within a restriction site. This mutation prevented Hind III from cutting between the 1- and 5-kb fragments. Therefore, the 6-kb fragment contains the region similar to the 1-kb fragment of the original. Thus, Hind III digestion may be used to distinguish between the four alleles above (in Lanes 2-5 of Figures 16-7B and 16-7C). This is the essence of RFLP analysis. Now the essential concepts necessary to understand RFLPs have been presented.

Methods

For RFLP analysis, sample DNA is first extracted, then digested with a restriction endonuclease. This digest is subjected to electrophoresis to separate the fragments. Detection of these fragments for visualization is achieved by means of a form of the Southern blot. Details of these steps are found below.

DNA Extraction

The initial step, in almost any molecular biology protocol, is to extract DNA. This particular step varies greatly among laboratories, but the principle is the same. First, the tissue is minced into single cells using a tissue grinder such as a mortar and pestle. In the case of blood, the leukocytes (white blood cells) are first separated from platelets and erythrocytes (red blood cells) and then washed free of blood plasma (liquid). Next, the nuclear DNA is freed from the cell nucleus by solubilizing the cell and nuclear membranes with a detergent such as SDS (sodium dodecyl sulfate).

For RFLPs, DNA is extracted and digested with a restriction enzyme, and fragments are separated by electrophoresis.

DNA extraction involves the degradation of proteins and the solubilizing of the cell membranes.

Chromosomes are found packed tightly by proteins. These proteins, as well as other cellular proteins, can be removed with a protease enzyme called *proteinase K* and a detergent and/or by phenol:chloroform treatment. The advantage of using a protease enzyme is that it also destroys nucleases that could cut the DNA. However, the protease must be removed or inactivated so that it doesn't degrade the restriction enzyme being used to digest the DNA. Chaotropic agents, such as guanidinium isothiocyanate, can be used to extract either DNA or RNA. This latter method is often used by commercial kits used in clinical laboratories. Next, the DNA is precipitated and concentrated with alcohol, and then pelleted by centrifuga-

tion and dimer. Newer methods use a saturated solution of sodium chloride (NaCl) to precipitate the cellular protein, followed by the precipitation of DNA with alcohol.

DNA concentration is determined by spectrophotometry or by dipsticks.

DNA concentration can be determined by measuring the absorbance of the rehydrated DNA in a spectrophotometer set within the ultraviolet range of light at 260 nanometers. This is the wavelength of light at which the nucleotide bases have maximal absorbance. In turn, the absorbance value can be converted into micrograms (ug) of DNA per milliliter (ml) of solution using a standard formula. (An absorbance value of 1 corresponds to approximately 50 ug/mL of DNA.) In lieu of spectrophotometry, colorimetric dipsticks are now available for estimating DNA concentration. This quicker method takes only a few minutes to perform. A resulting color change is compared to the color chart provided with the commercial kit.

DNA Digestion

It takes several hours to digest human chromosomal DNA using restriction enzymes.

Several hundred different restriction enzymes exist. DNA is digested with a restriction enzyme under the precise incubation temperature and pH specified for that particular endonuclease. These variables are usually detailed by the manufacturer that provides the enzyme. If two enzymes have the same requirements, double digests can be performed in the same reaction vial. Appropriate restriction enzymes will cut human chromosomal DNA into a million fragments in approximately three hours.

Electrophoresis

A transilluminator is used to view DNA fluorescence.

Digested DNA fragments are separated by size using gel electrophoresis (see Chapter 1). Agarose is nontoxic, unlike the alternative gel matrix, acrylamide, which is a neurotoxin. The concentration of agarose used to prepare a gel will depend on the size of the DNA fragments that are to be separated. Visualization can be accomplished by staining the gel either prior to pouring the melted agarose into the cast or after electrophoresis is complete. Usually ethidium bromide, an intercalating DNA-binding dye, is used, but those working with it should note that it is a *dangerous* mutagen that requires the use of gloves and special disposal procedures. (Just as the chemical binds to DNA bases on the gel, so can it bind to cellular DNA.) When the ethidium bromide gel is exposed to an ultraviolet light called a *transilluminator*, the DNA can

be seen as bright fluorescent regions. Because of the vast amount of DNA extracted from cells in this particular procedure (using total cellular DNA as opposed to a small piece of DNA), the cell DNA digests will appear as bright smears. The simultaneously run DNA size standards, however, will be seen as a ladder of discrete bands. By photographing the standards (usually put into Lane 1 for reference), one has a documented "ruler" to compare with the migration distances of the fragments seen by Southern blot (to be performed in the next step). The fragments will then be transferred to a membrane and probed to allow the visualization of those fragments of interest.

DNA Transfer and Southern Blotting

Either heat or a strong base solution can break hydrogen bonds and denature the DNA in the gel. In Southern blotting, the gel is usually submersed into an alkali (sodium hydroxide) to denature the DNA prior to transferring the fragments to the membrane. Southern transfer (blotting) of DNA from an agarose or an acrylamide gel to a membrane made of nylon or nitrocellulose is done so that the DNA fragments can be probed to enable the detection of riflips. This transfer is accomplished in one of several ways. The traditional method, named for its discoverer, E. M. Southern, is a capillary transfer method whereby the gel is placed under the membrane in a large dish. Absorbent paper towels are stacked on top of the membrane, and wicks made of paper are immersed into a buffer solution in the dish. The buffer moves up into a stack of paper towels, carrying the fragments into the membrane. This process is slow and usually requires overnight incubation. In some systems, the direction of migration can be inverted to allow fragments to move in a downward fashion. This decreases incubation time to several hours. Also, a vacuum system or electrical current (electroblotting) can be used to speed up the process. After the transfer, the membrane will be an exact replica of the gel, with no bands visible.

Nitrocellulose membranes are very fragile, and both types of materials (nitrocellulose and nylon) require handling around the edges only and with gloved hands. After transfer to the membrane, the DNA may be "fixed" (made to permanently attach) onto the membrane with ultraviolet light or by baking it in an oven.

Radioactive probes are detected by a technique called autoradiography.

Once the transfer has been completed, the next step requires a nucleic acid probe specific for the “gene” under study to be hybridized to the membrane. This is performed by placing the membrane in a hybridizing solution containing the marked (labeled) probe. The probe is a short piece of single-stranded complementary DNA (cDNA) conjugated to a marker or tag. The marker may be radioisotopic, but this can be expensive and hazardous. A radioisotope of phosphorus, ^{32}P (pronounced “P 32”) is commonly used. Such radioactive probes can be detected by a technique called *autoradiography*. With this technique, the membrane with the bound (radioactive) probe is placed on photographic paper used for X-rays. The radioactivity will develop the film at each site where the probe is bound, resulting in what is termed an *autoradiogram*. This whole process can take up to one week to perform. Newer markers for probes are nonisotopic (nonradioactive), less hazardous, and often faster and less expensive. The major difference between isotopic and nonisotopic labels is that, with ^{32}P , the radioactivity can be measured directly; whereas, when using a nonisotopic label, another molecule is required to measure the amount of bound probe. For example, the probe is often conjugated to an antibody which binds to an enzyme. After binding, the membrane is washed to remove excess probe-enzyme conjugate. Then the substrate for the enzyme is added to produce a colored product. This color can be seen on the membrane everywhere the probe has bound to the fragment. The locations of the fragments are then compared to the photograph of the molecular weight standards. After hybridization has occurred, the “blot” is removed and washed.

As an alternative to DNA transfer, DNA fragments can be eluted from the gel, placed into a tube with the probe-enzyme conjugate and the enzyme’s substrate, and read photometrically (i.e., in a spectrophotometer).

Polymerase Chain Reaction (PCR)

Southern blotting can be replaced with PCR.

The Southern blot assay is often complemented by or substituted for the polymerase chain reaction (see Chapter 15). PCR amplifies (copies) pieces of DNA, RNA, or a gene a million- to a billion-fold. Instead of a Southern blot, DNA fragments can be amplified by PCR, and the amplified fragments can be separated by electrophoresis. This would provide enough amplified DNA so that fragments could be seen by staining the gel.

Alternatively, one could add a drop of amplified fragments to a membrane and detect the region of interest by hybridization to specific probes (dot blotting).

DNA Sequencing

The most definitive way to determine polymorphism is to compare the base sequences between fragments generated from enzymatic digestion. At this time, however, this is not done routinely outside of the research laboratory (see Figure 16-8).

Polymorphism ultimately is determined by comparing base sequences between digested DNA fragments.

Polymorphism Revisited

Analysis of noncoding regions of DNA from homologous chromosome pairs reveals an average of one nucleotide difference in every 250 nucleotides. However, a particular DNA polymorphism must have a frequency greater than 1% within a population to be useful as a riflips. Currently there exists a library of useful probes for over 1000 existing ("known") riflips. These probes are available for research and clinical use.

There are two major classes of polymorphisms for which RFLP analysis takes advantage. The first class, *restriction site polymorphisms*, involves the loss or gain of a restriction site, similar to the substitution mutation seen in Figure 16-4A. This class is widely utilized in the detection of genetic diseases. The second class is similar to the examples seen in Lanes 4 and 5 of Figure 16- 5B. It has nothing to do with the actual recognition sequence but still affects the sizes of the digested fragments. This latter class is referred to as *minisatellite sequences with variable number tandem repeats* (VNTRs,) and it will be described in the forensics section since it is more commonly used in that discipline.

Restriction site polymorphism is used for the detection of genetic disease.

Specific Applications of RFLP Analysis

The enormous power of RFLP analysis is reflected in the diversity of its application. Since 1980, when the use of riflips as a genetic marker for sickle-cell anemia was introduced, the range of application for this method has expanded into forensics and epidemiology. Some of these applications are discussed in the following sections.

Detection of Genetic Diseases

RFLP analysis has been used in prenatal diagnosis since

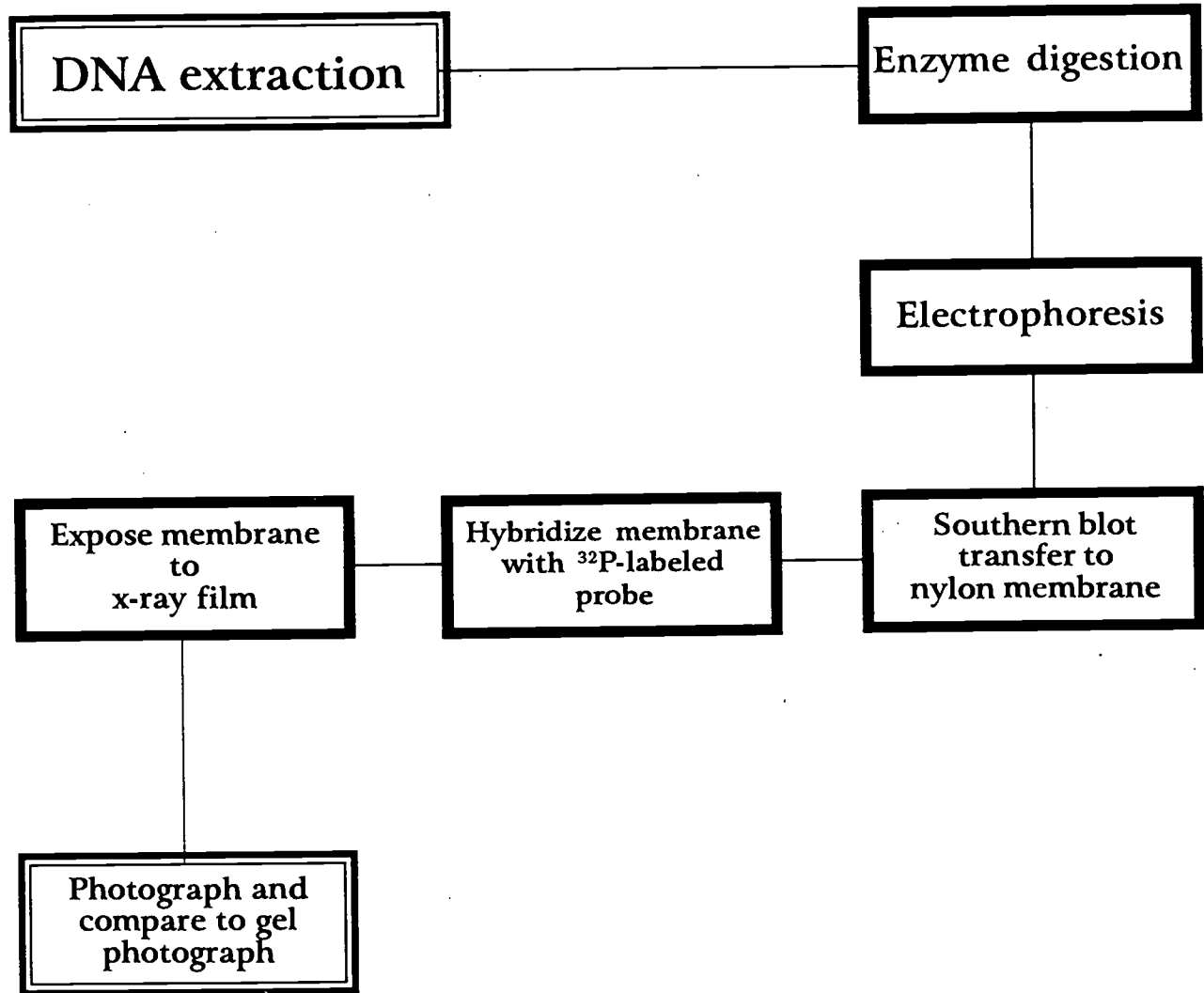


Figure 16-8

Comparing base sequence between fragments generated from enzymatic digestion.

312 Restriction Fragment Length Polymorphisms (RFLPs)

Polymorphisms found by the use of restriction enzymes can be used to detect genetic disorders.

the 1980s. Genetic testing is based on the fact that all the somatic cells of an individual (from conception) contain a full set of genetic instructions. More than 200 genetic disorders can be diagnosed using RFLP techniques. Polymorphisms found within restriction enzyme sites can be useful in the detection of genetic disease: (1) *if* they are located within a gene for a genetic disorder (*intragenic locus*) or (2) *if* they are closely-linked to the gene for a genetic disorder (found, closely-linked to the gene on the same chromosome). This is termed an *extragenic locus*.

RFLPs can be used to diagnose disease if the disease is caused by a mutation that directly changes a restriction site within a gene (*intragenic locus*). In this case, a new site is formed or a known site is no longer available when a patient has the gene for the disease. A good example of this is the sickle-cell mutation. The normal gene for B-globin contains a recognition sequence for the restriction enzymes Mst II and Cvn I. When cut, two fragments are formed that measure 1.15 kb (1150 base pairs) and 0.2 kb (200 base pairs). However, the single base substitution found in the sickle cell allele abolishes the Mst II recognition site. This results in a single fragment of 1.35 kb (1,350 base pairs) in size. This A to T substitution in the sickle cell allele is shown Figure 16-9. To locate the correct fragment after transferring the gel fragments to a nylon membrane, the blot is probed with a piece of DNA complementary to the gene itself. Such a probe should bind to both alleles, even the allele with the substitution.

For many diseases, we do not know the exact location of the gene causing the disease. In this indirect approach, RFLPs are used as "markers" for the disease (*extragenic locus*). Blood samples from relatives are analyzed for a polymorphism that seems to be co-inherited with the disease in question. This requires that a polymorphism be closely-linked to the disease locus in order to decrease the chance of the two loci being separated during a cross-over event. (Recall that during meiosis, the chromosome pairs find each other and synapse. During prophase I of meiosis, genetic information can be exchanged.) Refer to the pair of homologous chromosomes shown in Figure 16-10A. With Figure 16-10A, assume that the letters are genes, each with two alternate alleles, capital and lowercase. (These genes are all dimorphic, not polymorphic.) During the pairing of chromosomes in meiosis (prophase I), the two chromosomes will synapse and a crossover event may occur. This crossover event can occur anywhere from the A/a gene down the chromosome.

Figure 16-9.

The normal B-globin sequence is shown for comparison with the sickle cell gene sequence. *Note the substitution mutation in the sickle cell allele.*

Normal B-globin Gene: C C T G A G G A G

Sickle Cell Gene: C C T G T G G A G

Figure 16-10A.

Two Homologous Chromosomes.
The alleles for all of the genes are different.

Chromosome A	Chromosome a
A	a
B	b
C	c
D	d
E	e
F	f
G	g

Figure 16-10B.

Two Homologous Chromosomes After a Cross-over Event.
Alleles A - D (a - d) have become unlinked from alleles E - G (e - g).

Chromosome A	Chromosome a
A	a
B	b
C	c
D	d
e	E
f	F
g	G

Consider one in which it occurs between the D/d and the E/e as shown in Figure 16-10B. As a result of this cross-over, alleles A, B, C, and D have become unlinked from E, F, and G. This is the only way that D and E could have become unlinked and moved to different chromosomes — the event had to have occurred at that exact location. However, any cross-over event, occurring anywhere along the chromosome, would have unlinked A and G. Therefore, the likelihood of A and G becoming unlinked is greater than that of D and E or A and B. That is why it is necessary for a specific locus to be close to a disease gene before it can serve as a marker for that disease gene. It must be close enough to that gene to make unlinking less likely to occur.

In Figure 16-10B, allele B would serve as a marker for gene A because they would be co-segregating alleles in most cases. If offspring inherited the A allele, then at gene B/b, they probably would have the B allele. If the b allele is the genetic disorder, and they have the A allele, then it is less likely that they have the disease allele. [Of course a crossover event could occur in just the right spot between two close loci (like in the example above between the D and E). That defines this as a marker, not the actual gene.]

In this indirect RFLP analysis, human pedigrees are studied. If a linked marker is found in a fetus to be similar to the diseased parent or sibling, then that fetus is at high risk for the disease. The classic examples of such use of RFLPs are in the cases of Huntington's disease and Duchenne's muscular dystrophy. The reader is urged to read about these discoveries in an easy-to-read publication written for students of biology entitled *Genome* by Jerry E. Bishop. In the spirit of Lewis Thomas, this delightful book explains even the most difficult of molecular biology techniques to young scientists.

The use of RFLPs for the diagnosis of genetic disease can be summarized as follows. If a defective gene with a dominant phenotype co-segregates (is inherited/is linked) with a specific RFLP pattern, then any family member whose DNA contains a similar RFLP pattern is at high risk to express that disease. If a recessive phenotype co-segregates with the RFLP pattern, then the person is a high risk carrier. The level of risk depends on the closeness of the RFLPs to the disease gene.

RFLPs are commonly used to aid in the diagnosis of:

- 1) *cystic fibrosis*
- 2) *Duchenne's muscular dystrophy*
- 3) *polycystic kidney disease*
- 4) *sickle-cell anemia*
- 5) *hemophilia*
- 6) *Huntington's disease.*

Forensics

Forensics testing utilizes the second class of polymorphisms — those that have nothing to do with the actual restriction enzyme recognition sequences. These polymorphisms exist within what is known as *repetitive DNA* or *minisatellite DNA*. Many DNA sequences are repetitive, some very moderately and some highly repetitive. Some highly repetitive sequences can be found in clusters of tandem repeats, simple core sequences that are repeated over and over again in a linear fashion (continuous head-to-tail repetitions). Such tandem repeats are reported to make up almost 50% of the fruit fly's genome! It is thought that these sequences may serve a function for chromosome replication and in meiosis during crossing-over. One can imagine how tandem repeated sequences might allow chromosomes to bind together by creating large stretches of complementary bases. On the other hand, because these sequences are repeated so many times, the chromosomes are likely to misalign during pairing. This causes an unequal crossover resulting in variations between individuals in the number of times a "core" sequence is repeated. So much polymorphism is possible that these clusters of tandem repeats can be used to characterize individual genomes using RFLPs (DNA fingerprinting). Think of the chromosome as having numerous regions of insertions or deletions of a basic core sequence. Some of these repeated segments are repeated tandemly anywhere from 5 to > 50 times and have a core measurement of 10 to 100 bases long. Such repeats and the regions in which they are located are literally *HOT SPOTS* for polymorphism as detected by restriction enzyme digestion. Because individuals have different numbers of the repeating core unit, we call these minisatellites *variable number tandem repeats* (VNTRs).

Tandem repeats are simple core sequences that repeat over and over in a linear manner.

DNA fingerprinting uses tandem repeats to identify individual genomes.

Assume that a minisatellite has a length of 60 bases. This core unit of 60 bases is repeated 15 times in 50% of the population, 20 times in 30% of the population, 14 times in 8% of the population, 10 times in 5% of the population, 5 times in 5% of the population, and 4 times in 2% of the population. These frequencies of variations are all greater than 1%, so this polymorphism appears to meet the criterion for usefulness within a population. If the number of repetitions of the core unit on a chromosome could be determined, it would help identify an individual. Fortunately RFLP technology can accomplish this. The DNA can be cut into fragments with a restriction

enzyme, then the fragment sizes analyzed. A larger number of repeated core units results in a larger fragment size.

In a violent crime, blood, tissue, or semen from the perpetrator is usually left at the scene of the crime, and likewise tissue from the victims is often found on the clothes of the perpetrator. Historically, blood typing has been used to connect the tissues with their source. More recently, tissue typing has been used. However, these results can only *exclude* a suspect. There may be other people with that particular tissue type. DNA, on the other hand, can identify an individual through RFLPs because DNA sequences are *unique*, except in the case of identical twins. The FBI uses RFLP analysis to aid in sexual assault cases. Usually only three to five probes specific for VNTRs are needed for DNA fingerprinting. As an example, one can distinguish between a rape suspect's DNA and a victim's DNA using VNTRs to create RFLPs upon restriction enzyme digestion (see Figure 16-11A).

DNA can identify individuals using RFLPs analysis.

Assume that the arrowheads are restriction sites. One of the fragments from the suspect would be larger than the corresponding fragment from the victim. To tell the difference after electrophoresing these digested DNA fragments (remember that the gel of total cellular DNA would be too heavily concentrated to see; i.e., it would be smeared), a ^{32}P -labeled probe, which is complementary to the core region, could be used. After the strands were denatured, the probe would be hybridized to the single-stranded target DNA. Then by autoradiography, bands would be seen everywhere the core region was found. The Southern blot might look like Figure 16-11B. The suspect's hybridizing fragment (that containing the core segment) is larger than the victim's because of the higher number of core repetitions (higher VNTRs) in the suspect's DNA. Thus, minisatellites are useful in RFLP analysis. (Remember that both individuals would actually have two chromosomes with this region. If either were heterozygous for this VNTR number, then he or she would have two bands of different sizes, instead of a single band.)

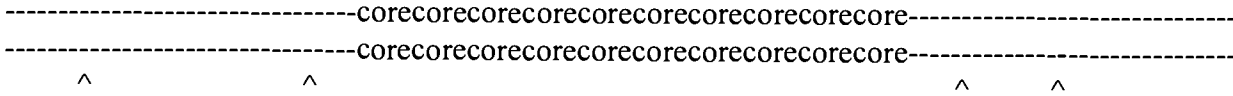
A useful family of minisatellites that shares a common core region, a GC-rich sequence of approximately 20 base pairs has been found in humans. This family is repeated thousands of times in the human genome — every 50 kb or so. Therefore, if a probe hybridizing to the core is used on digested DNA, the resulting Southern blot looks like a supermarket bar code. In the fictitious example in Figure 16-11B, we see how different a single

Figure 16-11A.

Chromosomes from the suspect and the victim.

Note the difference in the VNTRs between the two individuals. (The suspect has 10; the victim has 4.) The arrowheads represent restriction enzyme digestion sites.

Part of a chromosome from the rape suspect



Part of a chromosome from the victim

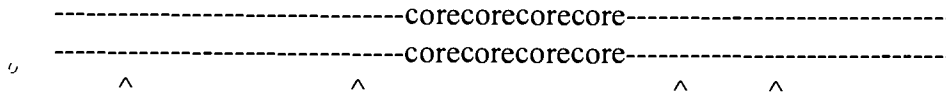


Figure 16-11B.

Autodiagrams of the suspect's and the victim's DNA.

On the left is a photograph of the molecular weight standards run simultaneously with the restriction enzyme digests of the suspect's and the victim's DNA. On the right is an autoradiograph of that gel using a sequence complementary to the core sequence as a probe. The suspect's fragment is heavier due to a higher number of VNTRs.

**Photograph of an
Agarose Gel**

Autoradiograph

Molecular weight
Standards

Suspect

Victim



core cluster can be between individuals. Just imagine that distinction a thousand fold. Now that's a fingerprint! The patterns are unique to every individual, except for identical twins. This is the basis of DNA fingerprinting. It is useful in criminology to identify the skeletal remains of a body or even for the identification of living persons who claim to be someone else.

It is estimated that, using DNA fingerprinting, there is only a 1 in 100,000 chance of two individuals having the same profile. It is not absolute, but it is still overwhelming evidence. The possibility of an identification is theoretically feasible when one takes into account that matches between different individuals at four and five probed loci have not been found in the FBI database. In paternity cases, with the use of just four DNA probes, it is possible to exclude paternity with a probability of 99.9% confidence.

Epidemiology

RFLPs can aid in the detection of hospital-acquired infections

RFLP analysis can be used for epidemiological evaluations of hospital outbreaks of infection. If several patients are found to get the same hospital-acquired infection (due to either a bacterium, virus, or fungus), the laboratory can compare drug susceptibilities between the two isolates. However, if these are identical, RFLP analysis can help to see if the two strains are genetically related. If they are, then a common source of contamination is sought.

Clinical Microbiology and Oncology

RFLPs can aid in distinguishing an infection from being pre-existing or new.

RFLPs are used in clinical laboratories in the genotyping of microbes to distinguish recurrent infections from new infection strains. In clinical virology, enzyme digestion patterns are useful in distinguishing between Herpes Simplex Virus I (HSV I) and HSV II. This is important because HSV I encephalitis is a more common sequela of the type I virus. RFLPs also help in typing papilloma viruses, some of which are associated with oncogenesis (cancer). This technique also has found its way into the clinical oncology laboratory for typing of tumors and assessing bone marrow transplants.

RFLPs are used in genetic studies.

Population Genetics

RFLP analysis has been shown to be helpful in population genetics studies of the Lesser Snow Goose and in passerine birds. Most recently, ethnologists have used RFLPs to focus on reproductive success within a species. Whereas documentation of maternity has always been fairly straightforward (although perhaps not for all species), the difficulty of paternity determination has been eased (somewhat) by the use of RFLP analysis.

Restriction Enzyme Mapping

RFLPs are used in the Human Genome Project

The Human Genome Project involves an international effort to map the human genome and to locate genetic markers (of which there are thousands) spaced throughout the chromosomes. This is made possible because of the abundance of RFLPs within the human genome. A restriction map is a linear sequence of sites separated by defined distances of DNA. For example, Figure 16-3A is a Hind III restriction map of the 27-kb piece of DNA. The actual construction of such a map requires several enzymes and probes to see how fragments are arranged on the chromosomes.

Future

RFLP analysis offers the advantage of high sensitivity, but it is labor intensive and very expensive. In addition, it takes a great deal of time (up to 2 weeks). For these reasons it is not used routinely, except when no other technique is available. PCR overcomes many of the problems described for RFLPs. With PCR, the fragments can be amplified in such a way that they can be visualized on a gel without the need for blotting; this is a time-saver. Plus, only a small amount of DNA is required if using PCR amplification. Such analyses can use even a single hair. PCR is also used in the analysis of genetic polymorphisms in mummified tissue or prehistoric material which is too degraded for conventional RFLP analysis. (Just for comparison, consider the following: Up to 10 nanograms of DNA are available from 1 milligram of bone. A shed hair yields 1-12 nanograms of DNA per root; a plucked hair, 1-750 nanograms per root. Whereas PCR only requires <1 nanogram of DNA, RFLP analysis requires 1 microgram of DNA for optimal results — 50 nanograms at the very minimum, if no mistakes are made.) Some laboratories are now choosing a newer technique, *ampli-*

Amplified fragment length polymorphism (AFLP) requires no enzymes to digest DNA.

fied fragment length polymorphisms (AFLP). AFLP requires no enzymes at all to cut the DNA. It also requires only crude extracts of DNA. AFLP is performed by amplifying regions between common genes. Those individuals with longer core repetitions (higher VNTRs) yield amplified fragments with a higher molecular weight.

One of the problems with RFLP analysis that is not solved by using PCR is the argument of whether or not our population actually is in a Hardy-Weinberg equilibrium. It is possible that many of the polymorphisms characterized for Caucasians may not be as polymorphic in other ethnic groups. This has been the legal argument against DNA typing. While there is little scientific dispute regarding the validity of RFLP analysis, there is extensive legal debate. Nevertheless, DNA typing is generally accepted by the courts. Other questions, such as, “Were the methods done properly?” and “Were the specimens handled properly?” are more commonly put forward for analyses involving PCR because of its innate sensitivity.

Conclusions

This chapter has touched upon a great deal of information in a limited space. It is important that the reader has read these details — not necessarily have total recall of them. The primary concern of the author is that you take away a sense of the abundance of polymorphisms within a population. Now, scientists can detect these polymorphisms at the DNA level. Many are not evident by phenotype but *are* evident upon RFLP analysis. Changes in DNA sequence affect restriction enzyme cutting sites and create RFLPs. Keep in mind that the RFLPs are not the mutations themselves — and that RFLPs are inherited just like genes. And, finally, as new techniques develop, RFLP analysis will be expanded.

Questions Most Commonly Asked by Students

Question 1: What keeps the bacterial cell from degrading its own DNA with the restriction enzyme it makes?

Bacteria protect their own DNA from their specific restriction endonuclease by a process called methylation, the addition of a methyl (CH₃)

group to the DNA. For example, an E. coli cell producing Eco RI methylates each Eco RI restriction site so that the palindrome is not recognized by the enzyme. Any other DNA entering the cell with an Eco RI restriction site that is not methylated will be cut.

Question 2. Should RFLP analysis be voluntary only or should an insurance company be allowed to require it? Have you considered your own genetic privacy?

No discussion of technology, particularly medical technology is complete without addressing the question, "We can, but do we want to?" It is projected that one day DNA fingerprints will be used as identification for making credit purchases and for driving a vehicle. Some states already are incorporating RFLPs into computer databases for convicted criminals. This may seem fine at first glance, but this information affects all of us on a personal level. Might not discrimination be a problem if one's profile reveals, say, a future of hypercholesterolemia, depression, or heart disease? RFLP analysis results could affect an individual's insurance coverage or career trajectory. And finally, is this information really useful in the case of a debilitating late onset disease, such as Huntington's Disease? These are questions which should be a part of any discussion on RFLPs.

Question 4: If a substitution mutation occurs in one strand of DNA what happens to the opposite strand?

During DNA replication, prior to cell division, the two strands of DNA are separated. Each serves as a template (mold) for the making of the complementary strand. Thus, one new strand would carry the mutation on to resulting progeny cell populations.



Dr. Matthews-Greer explains to Patti Bouillon, senior medical technology student at LSU Medical Center, School of Allied Health Profession, how to measure bands on an electrophoresis gel and how to operate the Techne Hybridization UVP Transilluminator oven.

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Some Internet Web Sites Related to Bioinstrumentation

Prepared by Mary Gail Yeates and James H. Wandersee
Louisiana State University, Baton Rouge, LA

URLs

<http://146.139.72.10/docs/ani/tpm/tpmdocs/AAEMStatus.html>

"Live cam" view of an electron microscope from Argonne National Laboratory. Updated every 15-20 minutes; has a status window which is an instrument panel from the instrument itself to let the viewer know what is happening.

<http://www.mwrn.com/subject/biologic.htm>

Biological applications of electron and light microscopy; on-line images of animals, plants, and microorganisms.

<http://www-sci.lib.uci.edu/HSG/MedicalPath.html#V1D>

Virology image database; award-winning web site.

<http://www.lmmb.ncifcrf.gov/EP/EPemail.html>

Two-dimensional gel electrophoresis databases. Lists many other web sites.

<http://rmc-www.lbl.gov/images/images.html>

Resource for molecular cytogenetics; images from Lawrence Berkeley National Laboratory.

http://www.science.smith.edu/chem/org_chem/ir/irspec.html

IR spectroscopy images created by college student Jocelyn Nadeau, Smith College '97. Click on a structure to see its spectrum.

<http://www.inus.com/DOC/isotope.htm>

Nice reference table of commonly used radioisotopes in biomedical science; gives isotope, atomic weight, radioactive half-life, and beta, gamma, and positron energies. A commercial site with links to sources such as the International Isotope Society.

<http://www.apollo.co.uk/a/pcr/>

Billed as the ultimate web page for PCR. Links on all aspects of PCR. Site is located in the UK.

<http://www.raven.umnh.utah.edu/umg/kits/kit.conservation/tablepage.html>

Shows results of protein electrophoresis presented in an attractive table format for easy comparison. Displays results for three proteins, as found in "mother, father, and baby plants a & b."

<http://149.156.87.70/3/intro.htm>

Answers the questions: (a) What is flow cytometry? and (b) How does it work? Site is based at the University of Massachusetts, in Amherst, MA.

<http://www.latrobe.edu.au/csi/csihome.html>

Center for Scientific Instrumentation's home page. Located at Latrobe University in Australia. Has Chromatography pages.

<http://www.bigeasy.com/~rytech/biology.html>

A high quality biology teaching resource site maintained by an energetic New Orleans, Louisiana high school biology teacher, Colleen Fiegel. Especially strong in links to microscopic images.

Note: Because a web site's contents may change dramatically and without notice, please visit these web sites yourself before directing your students to them.

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