

**Modern
Biology
Series 5**

**A Laboratory Course
in
Cell and Molecular
Biology**

**By
John N. Anderson**

Suggested Reading and References for Part B

Laboratory Methods:

Hames, B.D. and Richwood, D. *Gel Electrophoresis of Nucleic Acids*. Oxford: IRL Press, 1982.

Hacket, P.B., Fuchs, J.A. and Messing, J.W. *An Introduction to Recombinant DNA Techniques*. Menlo Park: The Benjamin/Cummings Publishing Co., 1984.

Maniatis, T., Fritsch, E.F. and Sambrook, J. *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor Laboratory Publications, 1982.

General References:

Alberts, B., Bray, L., Lewis, J., Raff, M., Roberts, R. and Watson. *Molecular Biology of the Cell*. New York and London: Garland, 1983.

Freifelder, D. *Molecular Biology*. Portola: Science Books Int., 1983.

Metzler, D.E. *Biochemistry*. New York: Academic Press, 1977.

Lewin, B. *Genes*. John Wiley and Sons, Inc.: New York, 1983.

PREFACE

This laboratory program originated from my desire to help students bridge the widening gap between traditional cell biology and molecular biology. To accomplish this objective, sections on biological theory are presented along with contemporary procedures. It is my hope that this combined approach will emphasize to the student that contemporary biology is a collection of techniques and concepts that are applied to explore fundamental biological principles.

The program consists of a series of laboratory experiments and can be used to teach 14 three-hour laboratory sessions. The chemicals and materials that are provided to perform these exercises are described in the Instructor Manual. In the first two sections of the course, students study contemporary topics in protein biochemistry including protein structure, function and isolation, and the detection and molecular basis of disease. In the third section of the course, students localize enzymes in plant and animal cells and study the properties of a specific cell-surface receptor. Students perform a project of their own design in the fourth section of the course. The projects focus on the characterization of plant peroxidases. Experiments on the properties and structure of DNA are presented in the final section of the course. These exercises stress the organization and complexity of the prokaryotic and eukaryotic genomes, gene function and regulation, and the structure of the eukaryotic chromosome. Each section begins with a discussion of basic principles which should be mastered by the student before the laboratory exercises are performed. The exercises consist of a background information section, an experimental procedure, and study questions. Before the laboratory, the student should read the background information, study the directions for doing the experiment and understand the reason for each step in the procedure. Students derive answers to the study questions after completing the exercise by integrating material found in the text with the results of the experiment.

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and destain the gels as described in Section IV.	
13. Add 20 μ l of sample buffer to each tube and incubate the tubes for 15 minutes at 37°C. This sample buffer contains RNase which serves to destroy RNA in the samples. The sample buffer also contains the detergent, Sarkosyl, which will disrupt the histone-DNA interactions, liberating the DNA in a form that can be analyzed by electrophoresis.	
14. The samples can then be stored at -20°C until the next laboratory session.	
Laboratory Session 2	
Electrophoretic Analysis of DNA	
1. Prepare 1.5% agarose gels as described in Section IV.	
2. Load 15 μ l of each sample into the sample wells as indicated below.	
Sample Well Number	
Tube 1	1
Tube 2	2
Tube 3	3
Dye Mixture	4
Tube 1	5
Tube 2	6
Tube 3	7
Dye Mixture	8
3. Electrophoresis until the orange-G has migrated to within 2 cm of the positive electrode end of the gel.	
4. Remove the gels from the unit, mark the positions of the marker dyes and stain	
11. Anatomy and Evolution of the Genome	
12. Properties of DNA	
13. Analysis of a Genomic Segment	
14. Cell Fractionation and DNA Isolation	
15. Genotype to Phenotype	
16. The Nucleosome Structure of Chromatin	

Procedure

Laboratory Session 1

Preparing the DNA Samples

The procedure is designed so that the preparation of the samples will be performed by the entire class as a group. However, there is sufficient buffer provided for 4 groups of students to conduct the experiment. Unless otherwise indicated, all steps should be performed at 4°C.

1. Place 4 g of tissue into a pre-cooled mortar and add 10 ml of pre-cooled nuclear incubation buffer.
2. Cut the tissue into small (about 1 mm²) sections with scissors and grind the sections with the pestle until a homogeneous suspension is formed. The mechanical action of the pestle as well as the chemical action of the detergent, NP-40, which is present in the buffer, will serve to disrupt the plasma membranes and to cause cell lysis.
3. Transfer this cell homogenate to a centrifuge tube and centrifuge at 4°C for 5 minutes at 500-1000 x G to pellet the nuclei.
4. Carefully pour off and discard the supernatant. Add 3 ml of nuclear digestion buffer to the nuclear pellet and mix well.
5. Divide the nuclear suspension into 3 approximately equal portions and place each portion into a small centrifuge tube located in an ice bath.
6. Label the tubes 1-3 with a waterproof marking pen.
7. Transfer tubes 2 and 3 from the ice bath to a 37°C incubator.
8. After 5 minutes, remove tube 2 from the incubator and return it to the ice bath.
9. After 30 minutes, remove tube 3 from the incubator and return it to the ice bath.
10. Centrifuge tubes 1-3 for 5 minutes at 500-1000 x G. Small chromatin fragments produced by the action of the nuclear enzymes, should be liberated into the supernatant fractions.
11. Carefully pour the supernatant from each tube into clean tubes labeled 1-3. These supernatants should contain soluble chromatin particles (mono-, di-, and tri-nucleosomes).
12. Each student or student pair should transfer 40 µl of these solutions into 3 small tubes provided with the Chemical Package.

BACKGROUND INFORMATION

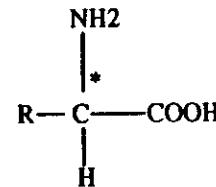
I. PROTEIN COMPOSITION AND STRUCTURE: A REVIEW OF THE BASICS

Proteins occupy a central position in the structure and function of all living organisms. Some proteins serve as structural components while others function in communication, defense, and cell regulation. The enzyme proteins act as biological catalysts which control the pace and nature of essentially all biochemical events. Indeed, although DNA serves as the genetic blueprint of a cell, none of the life processes would be possible without the proteins.

Amino Acids - Building Blocks of Proteins

The fundamental unit of proteins is the amino acid. The common amino acids have the general structure shown in Figure 1. Each amino acid has an amino group (NH_2) and a carboxylic acid group (COOH) attached to a central carbon atom called the alpha carbon. Also attached to the alpha carbon are a hydrogen atom and an R-group or side chain.

Figure 1. General Structure of Alpha-Amino Acids.



The C stands for a carbon atom; C* is the alpha carbon; H is hydrogen; N is nitrogen, O is oxygen, $-\text{NH}_2$ is an amino group and $-\text{COOH}$ is the carboxylic acid group. R is a general term for any one of several different side chains that determine the nature of different amino acids.

There are 20 amino acids commonly found in proteins and these differ from each other in the nature of the R-groups attached to the alpha carbon. A convenient classification of amino acids depends on the number of acidic and basic groups that are present. Thus, the neutral amino acids contain one amino and one carboxyl group. The acidic amino acids have an excess of acidic carboxyl over amino groups. The basic amino acids possess an excess of basic amino groups. Table I lists the major amino acids found in proteins.

Objective	To study the sequential breakdown of DNA in chromatin during incubation of isolated nuclei. This is an optional exercise that requires two laboratory sessions. In the first session, you will prepare nucleic acid digestes from chromatin. In the second session, you will use electrophoresis to analyze the DNA from this chromatin. This experiment is optional because a tissue must be provided for the preparation of nucleic acid.
Materials	A. The solutions and materials required for electrophoresis, sample handling and gel staining (see Appendices 1 and 2). B. The samples below are provided in the container marked Experiment 306:
Dye Mixture:	A mixture of xylene cyanol, bromophenol blue, and orange-G.
Materials Not Provided	Water Bath for Tube Incubation Maintained at 37 °C Ice Bath Refrigerated centrifuge and small (3-10 ml capacity) centrifuge tubes. A small clinical centrifuge placed in the refrigerator is suitable for this exercise.
	Four grams of tissue. Although nuclei from vertebrate liver contain large quantities of the desired enzyme, nuclei from other tissue sources can also be used. Possible tissues include: a. Calf or chicken liver obtained from animals recently butchered at your local slaughterhouse. b. Rodent liver, frog liver.
	There are sufficient materials provided with the Chemical Package for you to perform this exercise with more than one tissue type.

Peptide bonds are enclosed in the dotted boxes. The dotted circle shows how a peptide bond is formed with the production of H₂O.

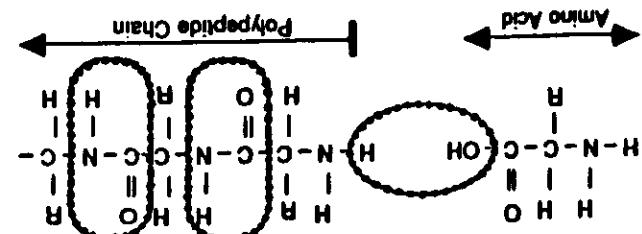


Figure 2. Formation of a Peptide Bond.

Proteins are composed of amino acids linked into chains by peptide bonds as shown in Figure 2. Two amino acids joined by a single peptide bond form a di-peptide; three amino acids form a tri-peptide, and a large number of amino acids joined together constitute a polypeptide. A protein is a polypeptide chain that contains more than 50-100 amino acids. The monomer units in the chain are known as amino acid residues. The average protein contains about 350 amino acids as amino acid residues. Although proteins and those with as few as 100 are not uncommon.

The Peptide Bond and the Primary Structure of Proteins

Neutral Amino Acids	Basic Amino Acids	Acidic Amino Acids	Aspartic Acid	Glutamic Acid
Glycine	Alanine	Arginine	Lysine	Hisidine
VaLINE	Leucine	Serine		
Isoleucine		Cysteine		
Threonine		Phenylalanine	Tyrosine	
Methionine				
Valine				

Table 1. Amino Acids Found in Proteins.

13. Add 20 µl of sample buffer to each tube and incubate the tubes for 15 minutes at 37°C. This sample buffer contains RNase which serves to destroy RNA in the samples. The sample buffer also contains the detergent, Sarkosyl, which will disrupt the histone-DNA interactions, liberating the DNA in a form that can be analyzed by electrophoresis.

14. The samples can then be stored at -20°C until the next laboratory session or analyzed by electrophoresis as described below.

Laboratory Session 2

Electrophoretic Analysis of DNA

1. Prepare 1.5% agarose gels as described in Section IV.
2. Load 15 µl of each sample into the sample wells as indicated below.

Sample Well Number	Sample
1	Tube 1
2	Tube 2
3	Tube 3
4	Dye Mixture
5	Tube 1
6	Tube 2
7	Tube 3
8	Dye Mixture

3. Electrophorese until the orange-G has migrated to within 2 cm of the positive electrode end of the gel.
4. Remove the gels from the unit, mark the positions of the marker dyes, and stain and destain the gels as described in Section IV.

C. Chromatin DNA Breakdown by Cell Nucleases (Optional)

The nuclei of most, if not all, eukaryotic cells contain a number of different nucleases. The physiological role of most of these enzymes is poorly understood. One such enzyme, or group of enzymes, is similar to micrococcal nuclease in that it attacks linker DNA between nucleosomes. The enzyme in rat liver nuclei has been characterized extensively. When liver nuclei are incubated at 37°C for various times up to 30 minutes, the progressive breakdown of chromatin occurs. DNA from this chromatin will exhibit the typical nucleosome "ladder" appearance upon electrophoresis. Although nuclei isolated from vertebrate liver contain large amounts of this enzyme, the enzyme activity is also found in nuclei from other animal and plant tissues.

The sequence or order of amino acids along a polypeptide chain is referred to as the primary structure of the protein. The primary structure of the protein myoglobin is given in Figure 3. This protein serves to bind and store oxygen in muscle. The primary structure of over 500 different proteins is now known.

Figure 3. The Primary Structure of Whale Myoglobin.

[Amino or N-terminus]

Val-Leu-Ser-Glu-gly-Glu-Trp-Gln-Leu-Val-Leu-His-Val-Tyr-Ala-Lys-Val-Glu-Ala-Asp-Val-Ala-Gly-His-Gly-Gln-Asp-Ile-Leu-Ile-Arg-Leu-Phe-Lys-Ser-His-Pro-Glu-Thr-Leu-Glu-Lys-Phe-Asp-Arg-Phe-Lys-His-Leu-Lys-Thr-Glu-Ala-Glu-Met-Lys-Ala-Ser-Glu-Asp-Leu-Lys-Gly-His-His-Glu-Ala-Glu-Leu-Thr-Ala-Leu-Gly-Ala-Ile-Leu-Lys-Lys-Gly-His-Glu-Ala-Glu-Lys-Leu-Lys-Pro-Leu-Ala-Gln-Ser-His-Ala-Thr-Lys-His-Lys-Ile-Pro-Ile-Lys-Tyr-Leu-Glu-Phe-Ile-Ser-Glu-Ala-Ile-Ile-His-Val-Leu-His-Ser-Arg-His-Pro-Gly-Asn-Phe-Gly-Ala-Asp-Ala-Gln-Gly-Ala-Met-Asn-Lys-Ala-Leu-Glu-Leu-Phe-Arg-Lys-Asp-Ile-Ala-Ala-Lys-Tyr-Lys-Glu-Leu-Gly-Tyr-Gln-Gly

This protein consists of 153 amino acid residues. Each residue in the protein is given a 3-letter abbreviation (thus: lysine = Lys; valine = Val). Proteins are always written with the free amino or N-terminus toward the left.

Three-Dimensional Protein Structure

In the cell, the polypeptide chain is folded into a highly ordered shape or conformation. Most proteins are globular in shape and these proteins are usually soluble in water or in aqueous media containing salts. This group includes the enzymes, antibodies, and a variety of other proteins. Less frequently, proteins are long and fibrous and most of these elongated molecules are insoluble in water and serve a role in the maintenance of cell structure.

The three-dimensional structure of a protein is due to the type and sequence of its constituent amino acids. Since the amino acid sequence of each protein is unique, it follows that different proteins assume different shapes. Thus, there is a remarkable diversity of three-dimensional protein forms. The conformation of a protein is usually of critical importance in the protein's function. For example, a protein can be unfolded into a polypeptide chain that has lost its original shape. In general, proteins such as enzymes are rendered nonfunctional upon unfolding because functional activity is dependent on the protein's native shape. This process is called denaturation. Most proteins can be denatured by heating, by certain detergents, and by extremes of pH. The ionic detergent, sodium dodecyl sulfate (SDS), is often used to denature proteins. The denaturing treatment can frequently be reversed, for example by removing the detergent or by neutralizing the pH.

B. Structure of Plant Chromatin

The wheat kernel consists of three parts: (1) The embryo or germ that produces the new plant; (2) the starchy endosperm, which serves as a food source for the embryo; and (3) covering layers which protect the grain. The average wheat grain is composed of about 85% endosperm, 13% covering layers and 2% germ, by weight. The endosperm is the raw material for flour production, and the wheat germ is used in food products as a source of vitamins. Wheat germ is also frequently used in the molecular biology laboratory as a source of protein synthesizing extracts as well as plant cell proteins and nucleic acids. In today's laboratory, you will analyze the structure of wheat germ chromatin.

Objective

To isolate nuclei from wheat germ, to digest the nuclear chromatin with micrococcal nuclease and to analyze the chromatin DNA by electrophoresis. This exercise requires two sessions of two hours or one session lasting three to four hours.

Materials

A. The solutions and materials required for electrophoresis, sample handling and gel staining (see Appendices 1 and 2).

B. The samples below are provided in the container marked **Experiment 306**:

Nuclear incubation buffer: This buffer should be made up before the laboratory session as described in Appendix 2 and stored in the refrigerator,

Sample buffer,

Dye mixture: A mixture of xylene cyanol, bromophenol blue, and orange-G,

Wheat germ,

Micrococcal nuclease: The enzyme should be made up before the laboratory session as described in Appendix 2 and stored in the refrigerator,

Cheese cloth,

Graduated pipets.

Materials Not Provided

A water bath for tube incubation maintained at 37°C.

Ice bath.

Refrigerated centrifuge and small (10 ml capacity) centrifuge tubes (A small clinical centrifuge placed in the refrigerator is suitable for this exercise).

Four small beakers.

Secondary Structure

The spatial arrangement of the protein backbone that is generated from the folding of the polypeptide chain is called the secondary structure of the protein. The secondary structures of proteins are stabilized by hydrogen bonds in which a hydrogen serves as a bridge between oxygen and nitrogen atoms (-C=O—HN-). A common secondary structure is the α -helix which consists of a single polypeptide chain coiled into a rigid cylinder. In the α -helix, each peptide bond along the polypeptide is itself hydrogen bonded to other peptide bonds. Many enzymes contain small regions of α -helices, while long sections of the α -helix are often found in proteins involved in cell structure. Another type of secondary structure of proteins is the β -sheet, which is a central organizing feature of enzymes, antibodies, and most other proteins that perform nonstructural functions. Here, a single polypeptide chain folds back and forth upon itself to produce a rather rigid sheet. Hydrogen bonds between neighboring polypeptide chains are a major stabilizing force for the β -sheet conformation.

Tertiary Structure

The tertiary structure of a protein describes the detailed features of the three-dimensional conformation of the polypeptide chain. It is brought about by the interactions between the amino acid side chains which cause the folding and bending of α -helix and β -sheet segments of the protein. One very important interaction at this level of organization involves the hydrophobic and hydrophilic side chains of the amino acid residues. Hydrophobic amino acids, such as phenylalanine and leucine, show limited solubility in water. Thus, these hydrophobic residues in a protein tend to cluster on the inside of the protein in order to avoid contact with the aqueous environment. Hydrophilic amino acids such as glutamic acid and lysine are readily soluble in water, and thus these amino acids arrange themselves on the surface of the protein molecule, where they can interact with water and with other hydrophilic side chains. The consequence of these interactions is that a polypeptide chain typically folds spontaneously into a stable, usually globular structure, with the hydrophobic side chains packed into the central core of the protein and the hydrophilic side chains forming the irregular, external surface.

Quaternary Structure

Some proteins contain more than one polypeptide chain. For example, each molecule of human hemoglobin consists of four polypeptide chains which are held together by a variety of noncovalent bonds. The arrangement of the polypeptides in such proteins is called the quaternary structure.

- These three DNA samples were prepared from calf thymus nuclei, as outlined in Figure 6-4 using low (nucleosome 1), medium (nucleosome 2) and high (nucleosome 3) concentrations of micrococcal nuclelease.
1. Prepare 1.5% agarose gels as described in Section IV and Experiment 301 and load 15 μ l of the following samples into the sample wells.
 2. Seal the wells with agarose and electrophoreses until the range-gels migrate to within 2 cm of the positive electrode end of the gel.
 3. Remove the gels from the unit, mark the positions of the marker dyes with a razor blade and stain and destain the gels as described in Section IV.
 4. Measure the distance of the DNA bands (in cm) from the sample origin and note the positions of the DNA bands.

Study Questions

1. Identify the DNA bands derived from one nucleosome (mononucleosomes), two nucleosomes (dinucleosomes) and three nucleosomes (trinucleosomes).
2. Note that the mononucleosome bands become slightly smaller with increasing

micrococcal nuclelease digestion. Why? (Hint: see Figure 6-3).

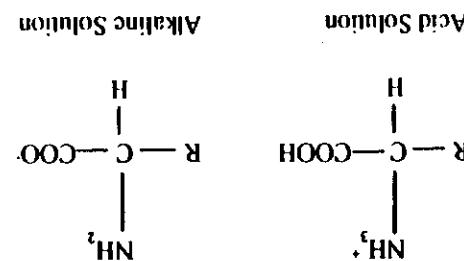


Figure 5. Amino Acids in Acid and Alkaline Solutions.

All amino acids contain at least one amino and one carboxyl group. In acid solutions, the amino groups are positively charged while the carboxyls are negatively charged and migrate in an electric field to the negative electrode. In basic solutions, the carboxyls are negatively charged while the amino groups are positively charged. In alkaline solutions, the carboxyls are deprotonated and migrate in an electric field to the positive electrode. In strong acid solutions, amino acids are not ionized. In alkaline solutions, they are ionized. In strong alkaline solutions, amino acids are negatively charged. In follows then, that in strong alkaline solutions, amino acids are negatively ionized, the carboxyls are negatively charged while the amino groups are positively charged and migrate in an electric field to the negative electrode. In basic solutions, amino acids are positively charged and migrate in an electric field to the positive electrode. In strong acid solutions, the carboxyls are positively charged while the amino groups are negatively charged (Figure 5). Therefore, in strong acid solutions, amino acids are positively charged and migrate in an electric field to the positive electrode. In basic solutions, amino acids are negatively charged and migrate in an electric field to the negative electrode.

Separation of Amino Acids and Proteins by Electrophoresis

A diagram of the essential components of an agarose electrophoretic system is shown in Figure 6. The agarose gel, containing protein sample wells, is submerged in buffer which is contained in the electrophoretic chamber. Samples to be separated are then loaded into the sample wells. Current from the power supply travels to the negative electrode (cathode), supplying electrons to the conductive buffer solution, gel, and positive electrode (anode), thus complicating the circuit.

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Sample Well Number	Sample	Dye Mixture	DNA Sample from Nucleosome Tube 1	DNA Sample from Nucleosome Tube 2	DNA Sample from Nucleosome Tube 3	DNA Sample from Nucleosome Tube 4	Dye Mixture	DNA Sample from Nucleosome Tube 5	DNA Sample from Nucleosome Tube 6	DNA Sample from Nucleosome Tube 7	DNA Sample from Nucleosome Tube 8
1	DNA Sample from Nucleosome Tube 1	DNA Sample from Nucleosome Tube 2	DNA Sample from Nucleosome Tube 3	DNA Sample from Nucleosome Tube 4	DNA Sample from Nucleosome Tube 5	DNA Sample from Nucleosome Tube 6	Dye Mixture	DNA Sample from Nucleosome Tube 7	DNA Sample from Nucleosome Tube 8	Dye Mixture	DNA Sample from Nucleosome Tube 1
2	DNA Sample from Nucleosome Tube 1	DNA Sample from Nucleosome Tube 2	DNA Sample from Nucleosome Tube 3	DNA Sample from Nucleosome Tube 4	DNA Sample from Nucleosome Tube 5	DNA Sample from Nucleosome Tube 6	Dye Mixture	DNA Sample from Nucleosome Tube 7	DNA Sample from Nucleosome Tube 8	Dye Mixture	DNA Sample from Nucleosome Tube 1
3	DNA Sample from Nucleosome Tube 1	DNA Sample from Nucleosome Tube 2	DNA Sample from Nucleosome Tube 3	DNA Sample from Nucleosome Tube 4	DNA Sample from Nucleosome Tube 5	DNA Sample from Nucleosome Tube 6	Dye Mixture	DNA Sample from Nucleosome Tube 7	DNA Sample from Nucleosome Tube 8	Dye Mixture	DNA Sample from Nucleosome Tube 1
4	DNA Sample from Nucleosome Tube 1	DNA Sample from Nucleosome Tube 2	DNA Sample from Nucleosome Tube 3	DNA Sample from Nucleosome Tube 4	DNA Sample from Nucleosome Tube 5	DNA Sample from Nucleosome Tube 6	Dye Mixture	DNA Sample from Nucleosome Tube 7	DNA Sample from Nucleosome Tube 8	Dye Mixture	DNA Sample from Nucleosome Tube 1
5	DNA Sample from Nucleosome Tube 1	DNA Sample from Nucleosome Tube 2	DNA Sample from Nucleosome Tube 3	DNA Sample from Nucleosome Tube 4	DNA Sample from Nucleosome Tube 5	DNA Sample from Nucleosome Tube 6	Dye Mixture	DNA Sample from Nucleosome Tube 7	DNA Sample from Nucleosome Tube 8	Dye Mixture	DNA Sample from Nucleosome Tube 1
6	DNA Sample from Nucleosome Tube 1	DNA Sample from Nucleosome Tube 2	DNA Sample from Nucleosome Tube 3	DNA Sample from Nucleosome Tube 4	DNA Sample from Nucleosome Tube 5	DNA Sample from Nucleosome Tube 6	Dye Mixture	DNA Sample from Nucleosome Tube 7	DNA Sample from Nucleosome Tube 8	Dye Mixture	DNA Sample from Nucleosome Tube 1
7	DNA Sample from Nucleosome Tube 1	DNA Sample from Nucleosome Tube 2	DNA Sample from Nucleosome Tube 3	DNA Sample from Nucleosome Tube 4	DNA Sample from Nucleosome Tube 5	DNA Sample from Nucleosome Tube 6	Dye Mixture	DNA Sample from Nucleosome Tube 7	DNA Sample from Nucleosome Tube 8	Dye Mixture	DNA Sample from Nucleosome Tube 1
8	DNA Sample from Nucleosome Tube 1	DNA Sample from Nucleosome Tube 2	DNA Sample from Nucleosome Tube 3	DNA Sample from Nucleosome Tube 4	DNA Sample from Nucleosome Tube 5	DNA Sample from Nucleosome Tube 6	Dye Mixture	DNA Sample from Nucleosome Tube 7	DNA Sample from Nucleosome Tube 8	Dye Mixture	DNA Sample from Nucleosome Tube 1

Electrophoresis is the movement of charged molecules under the influence of an electric field. Because amino acids and proteins are charged molecules, they migrate in an electric field at appropriate pH values. In the most common form of electrophoresis, the sample is applied to a stabilizing medium which serves as a matrix for the buffer in which the sample molecules travel. The agarose gel is usually used in the buffer in which the sample molecules travel. The agarose gel is large enough to allow even the smallest proteins to pass undispersed.

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General Description

II. Theoretical Aspects of Electrophoresis

Figure 6-4. Analysis of DNA from Micrococcal-Nuclease-Digested Nuclei

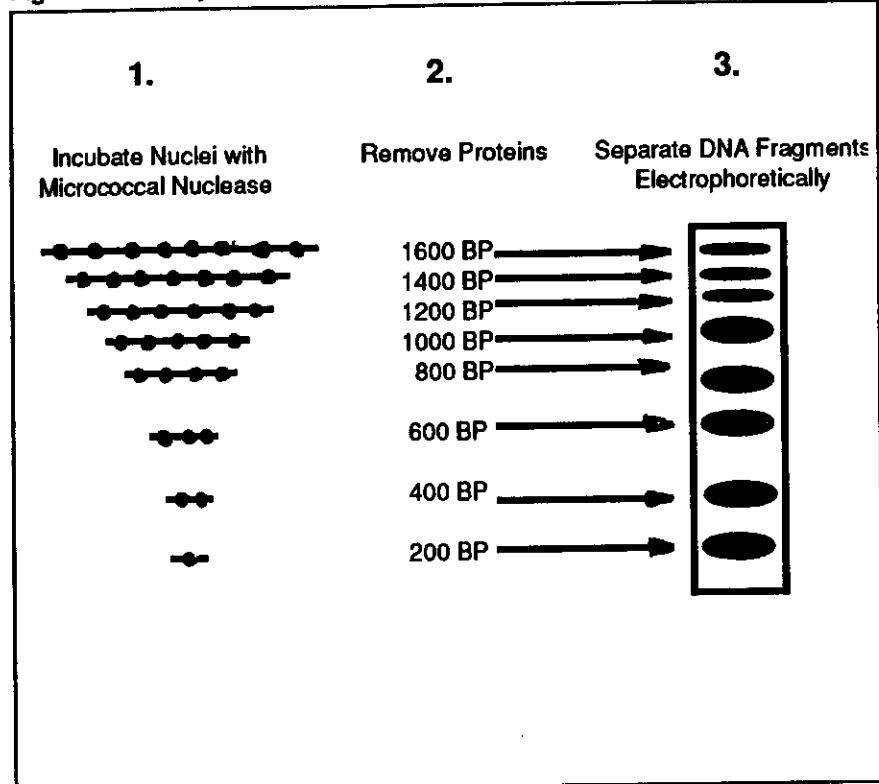
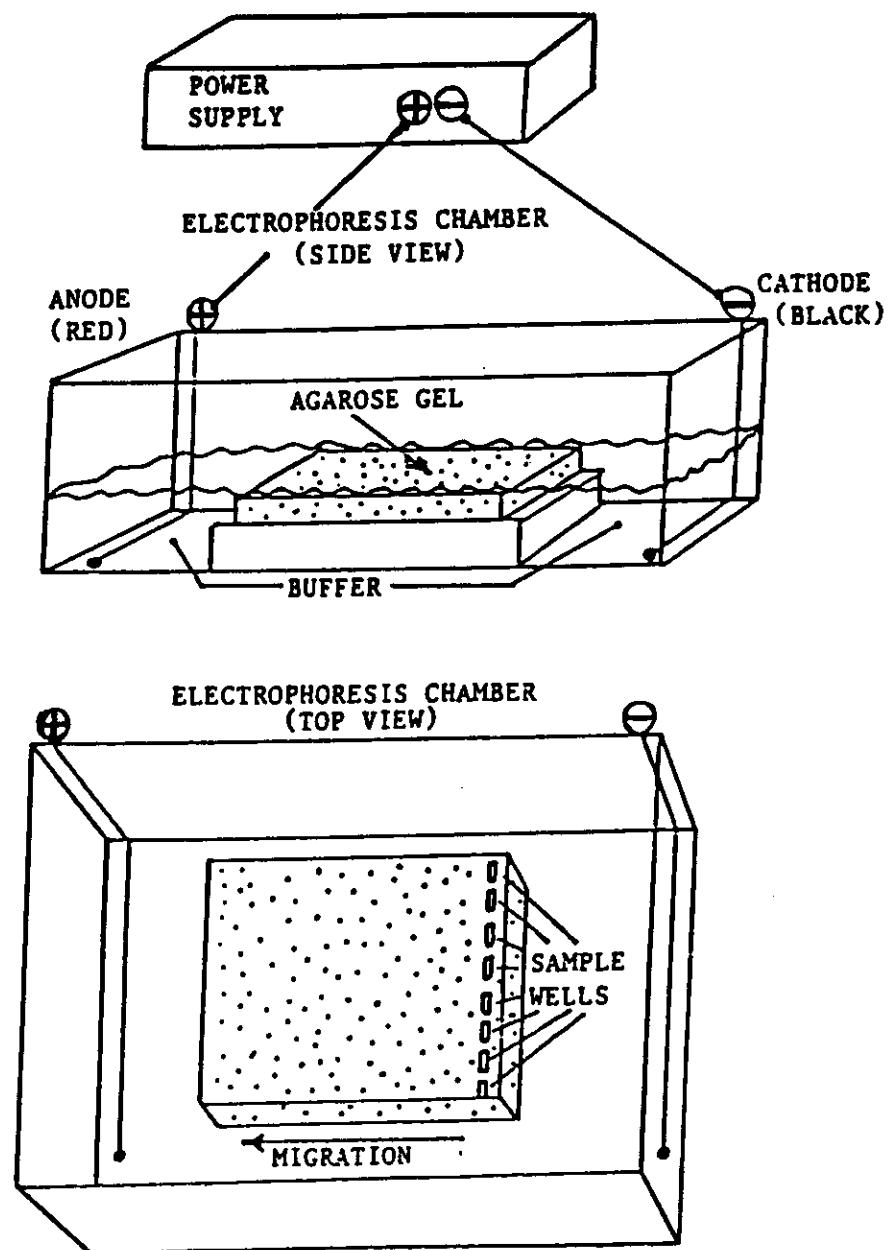


Figure 6. Components of a Horizontal Electrophoresis System.



Objective

To examine DNA obtained from micrococcal-nuclease-digested nuclei. The digested DNA is provided in the Experiment Package. The nuclei were obtained from calf thymus. In Parts B and C (below) you will analyze nucleosomal DNA in nuclei that you prepare.

Materials

A. The solutions and materials required for electrophoresis, sample handling and gel staining (see Appendices 1 and 2).

B. The samples below are provided in the container marked **Experiment 306**:

Dye mixture: A mixture of xylene cyanol, bromophenol blue, and orange-G,
 Nucleosome 1,
 Nucleosome 2,
 Nucleosome 3.

8

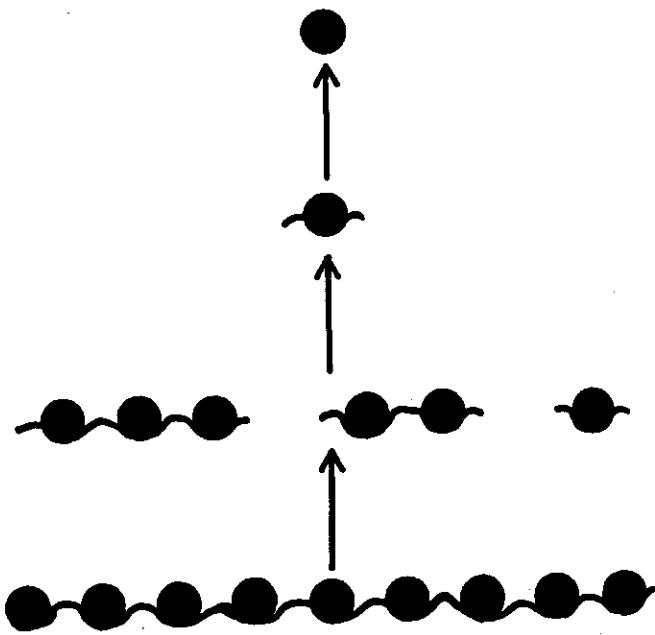


Figure 6-3. Sequential Breakdown of Chromatin by Micrococcal Nuclease

Additional evidence for the nucleosome model of chromatin is derived from nucleic acid digestion studies. A nucleic acid that breaks down DNA or RNA. Micrococcal nuclease is a bacterial enzyme that is often used for chromatin studies. This enzyme, unlike restriction nucleases, does not recognize a specific DNA sequence, but cleaves DNA that is not protected from digestion by proteins such as nucleosomal core histones. When nucleoli are isolated and incubated with this nucleosomal core histones, the enzyme crosses the nuclear membrane and cleaves the nucleosomal nucleic acid. When nucleoli are isolated and incubated with this nucleosomal core histones. A nucleic acid that is an enzyme that breaks down DNA or RNA. Micrococcal nuclease is an enzyme that is often used for chromatin studies. This enzyme, unlike restriction nucleases, does not recognize a specific DNA sequence, but cleaves DNA that is not protected from digestion by proteins such as nucleosomal core histones. When nucleoli are isolated and incubated with this nucleosomal core histones, the enzyme crosses the nuclear membrane and cleaves the nucleosomal nucleic acid.

The Procell Horizontal Electrophoresis unit is composed of an acrylic cell with central platinum electrodes, four removable gel casting trays, four sample wells forming combs and a safety lid with power cords. The four gels are made in the casting trays and then placed on the central platform of the electrophoresis cell. Each gel contains 8 separate sample wells. The experiments described below were designed such that each student uses four sample wells per experimental run. If the students work in pairs, the system can be used by 16 students.

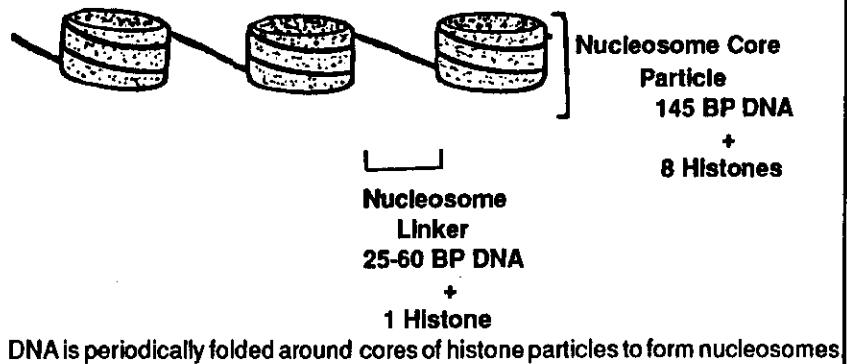
Electrophoresis Equipment

III. PRACTICAL ASPECTS OF ELECTROPHORESIS

The positively and negatively charged side chains of proteins cause them to migrate like amino acids in an electric field. The electrochemical character of a protein is dependent primarily on the numerous positively charged ammonium groups (NH_3^+) or aspartic acid and glutamic acid. The isoelectric points of most proteins is in the range of pH 5 to 7. Electrophoresis of proteins is usually performed at a pH above the isoelectric point of most proteins. The pH of the electrophoresis buffer used in the exercise described in this manual is 8.6. Thus, at pH 8.6 most proteins are negatively charged and when applied to sample wells at the negative electrode end of the gel, they travel towards the positive electrode. The rate of migration of a protein species in an electric field depends upon its net charge; the higher the charge the faster the protein will travel. For example, serum albumin, which has an isoelectric point of 4.8, will carry a strong negative charge in a buffer of pH 8.6 as compared to myoglobin, which has an isoelectric point of 7.2. Therefore, at pH 8.6 albumin will migrate toward the positive electrode at a much faster rate than myoglobin. These considerations form the basis for the electrophoretic separation of proteins according to net charge.

Thus there must be an intermediate pH at which each amino acid bears no net charge and does not migrate in an electric field. The pH at which an amino acid or protein does not migrate in an electric field is called the isoelectric point. Most neutral amino acids have isoelectric points around pH 6.0. The isoelectric points of acidic amino acids carry a negative charge at pH 6, whereas aspartic acid and glutamic acid, however, are close to pH 3. Therefore, at pH 6, these acidic amino acids carry a positive charge and migrate to the positive electrode during electrophoresis. The isoelectric points of the basic amino acids, lysine and arginine, are pH 9.7 and 10.8, respectively. These amino acids carry a positive charge at pH 6, and hence migrate to the negative electrode. These differences in charge permit the electrophoretic separation of acidic, neutral, and basic amino acids at pH 6, as illustrated in Figure 7.

Figure 6-1. The Nucleosome Structure of Chromatin



There are two major lines of evidence for the nucleosome model of chromatin. The first comes from studies of the unfolded chromatin fiber observed with the electron microscope (Figure 6-2). When the nucleus of essentially any eukaryotic cell is broken open and its chromatin content examined, electron microscopy reveals a "beads-on-a-string" structure. Each nucleosome "bead" is a disc-shaped particle with a diameter of about 10 nanometers.

Figure 6-2. Electron Micrograph of Chromatin Strands

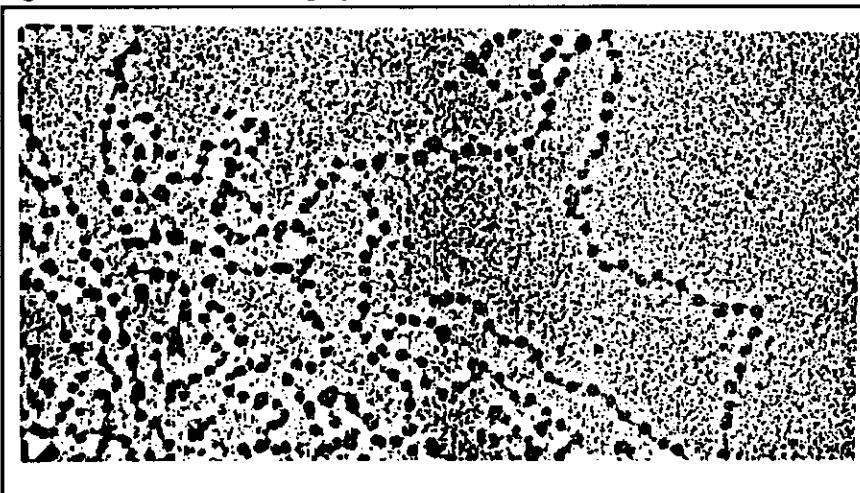
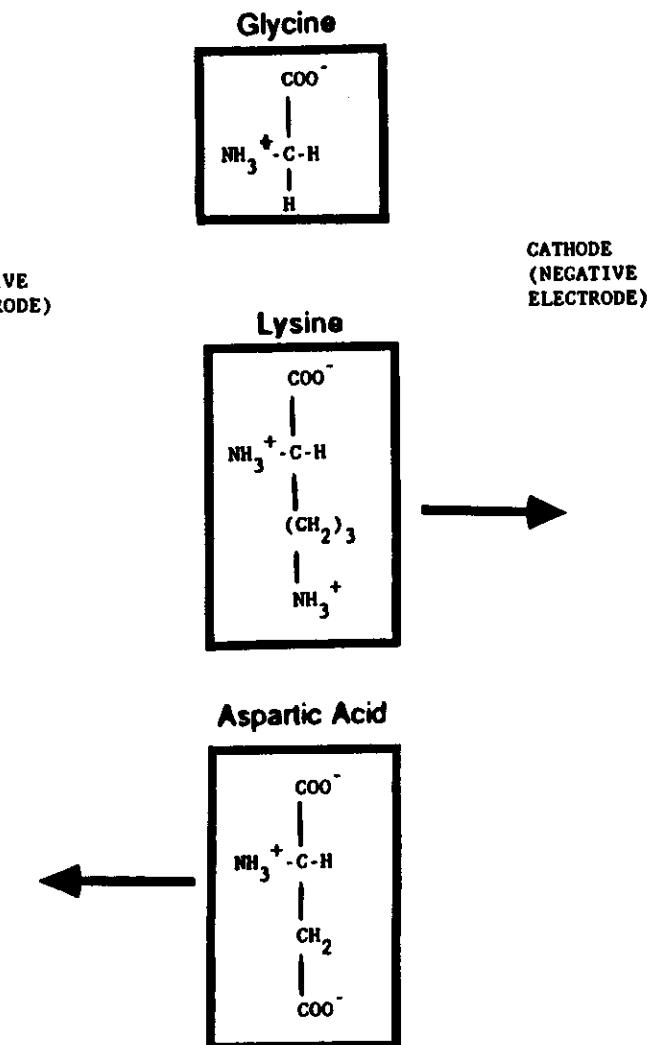
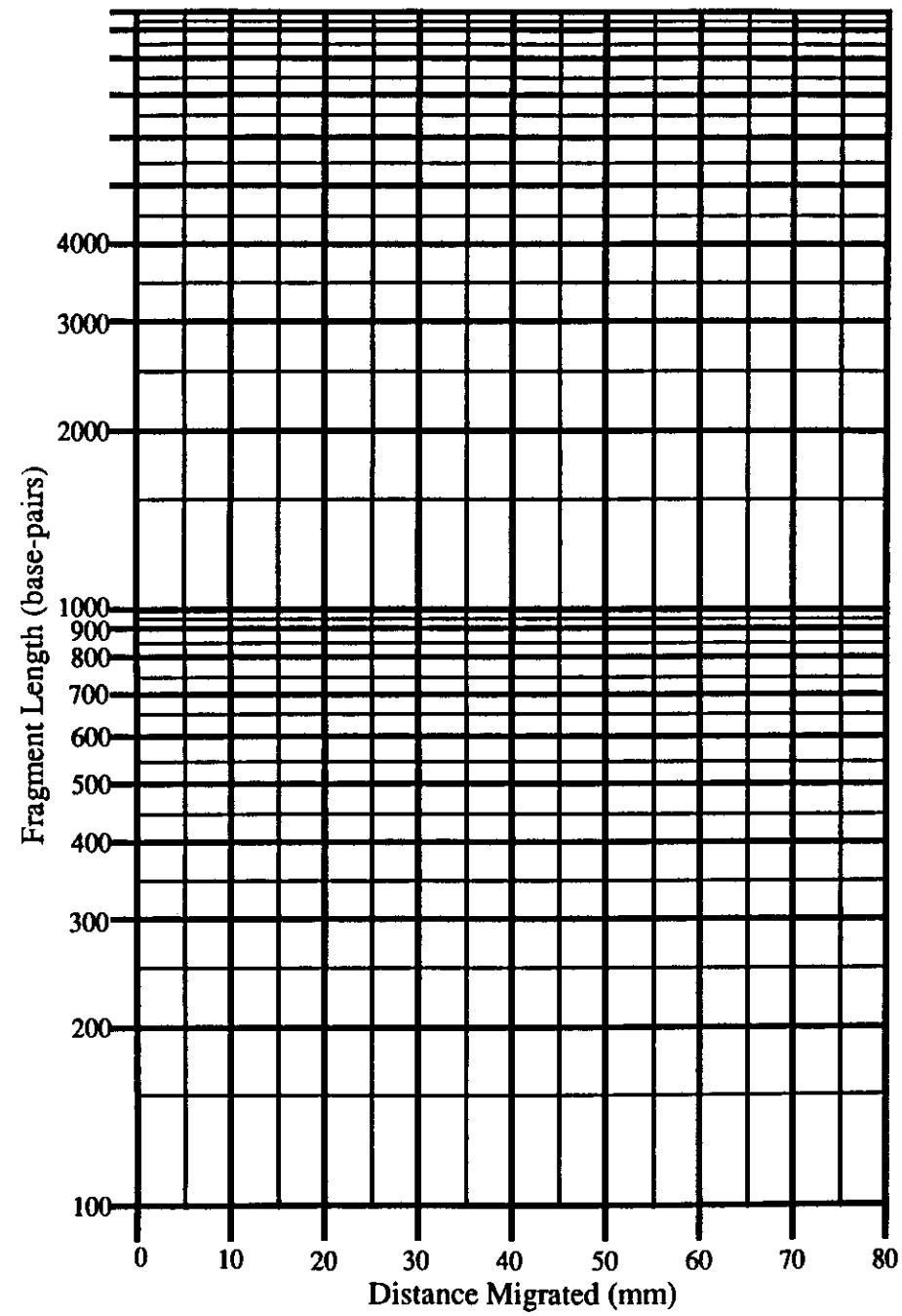


Figure 7. Separation of Amino Acids by Electrophoresis.

At pH 6, the basic amino acid, lysine, is a cation and moves to the cathode during electrophoresis. Aspartic acid is an anion at this pH and migrates to the anode. Glycine is not charged and hence does not migrate in the electric field. Paper strips are often used as a support medium for the electrophoretic separation of amino acids.





Staining and Destaining

Most proteins are not colored, and therefore it is necessary to visualize them in some way in order to determine their position in the agarose gel after electrophoresis. The most commonly used stain for the detection of proteins is Coomassie blue, and this stain has been incorporated into the staining solution that you will use. The staining solution also contains acetic acid which serves to precipitate and immobilize the proteins in the structure of the gel matrix after electrophoresis. The acid serves to fix the proteins in the gel so that the protein bands do not become blurred by diffusion. After the proteins in the gel have been stained, the unbound dye must be rinsed from the gel by a process known as destaining. A dilute solution of acetic acid and methanol is often used for the destaining of the agarose gels.

The Sample Buffer

The protein samples in the exercises described below are loaded into the wells of the agarose gel as 10-20% glycerol solutions. The viscous glycerol ensures that the samples will layer smoothly at the bottom of the sample wells. The sample buffer also contains the tracking dye bromophenol blue. As will be described below, this dye enables the investigator to follow the progress of an electrophoretic run.

Accessories

The accessories listed below are required to perform the experiments described in this manual. They are used for sample handling, and for the preparation and analysis of the agarose gels.

- *Glass test tubes (25ml)
- *Micro tubes (0.5ml)
- *Tube holders for the 0.5ml tubes
- *Tape

- *Gloves
- *Gel staining trays with lids
- *Macropipets (pipet - syringe)
- *Micropipetors and micropipets

- Number two small (0.5ml) tubes according to the plasmid letters (A, B, or C) that you used for transformation.
- Place 10µl of the EcoR1-buffer solution into each tube.
- Add 5µl of the corresponding plasmids to the tubes. Gently tap the tubes with the tip of your index finger to mix the solutions. Incubate the tubes for 60 minutes at 37°C.
- During this incubation, prepare 1.2% agarose gels as described in the first part of this manual.
- At the end of the 60 minute incubation period, add 5µl of electrophoresis sample buffer to each tube.
- Load 15µl of the following samples into the sample wells.

Sample Well	Sample
1	Tube A, B, or C
2	
3	
4	DNA Markers*
5	Tube A, B, or C
6	
7	
8	DNA Markers*

*The sizes of these DNA markers are: 784, 1120, 2040, and 3621 base pairs.

- Electrophorese until the bromophenol blue in the samples has migrated to within 1mm of the positive electrode end of the gel.
- During the electrophoretic run, return to Part A of this exercise (Step II - 5) and complete the bacterial transformation.
- At the end of the electrophoretic run, remove the gels from the unit and stain them as described in the first part of this manual.

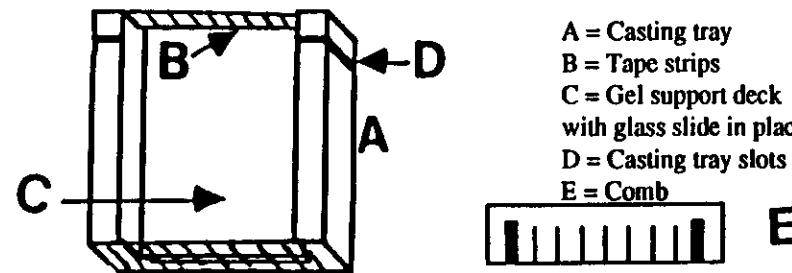
IV. PROCEDURES FOR THE PREPARATION, ELECTROPHORESIS AND STAINING OF AGAROSE GELS

The exercises described in this manual were designed such that the samples of two students are analyzed on one agarose gel. If students work in pairs, four students will share one gel. Four agarose gels are electrophoresed simultaneously using the Procell and MB-170 power supply.

Pouring the Agarose Gels

- Place the casting tray on a level work surface and place a precleaned glass slide into the gel support deck.
- Seal both ends of the gel support deck with tape. The tape must be firmly pressed against the edges of the deck to ensure a tight seal.

CASTING TRAY ASSEMBLY



A = Casting tray
B = Tape strips
C = Gel support deck with glass slide in place
D = Casting tray slots
E = Comb

- * With the macropipetor (pipet-syringe), dispense 15 ml of electrophoresis buffer into a 25ml glass test tube and add 0.18 grams of agarose. The agarose can be weighed out directly on an appropriate balance. If a balance is not available, 0.18 grams of agarose can be estimated by filling a 0.5ml micro tube with agarose until two-thirds full. Gently swirl the glass tube until the agarose forms a suspension.

- Place the test tube into a boiling water bath and allow the agarose suspension to come to a vigorous boil. After boiling for about one minute, remove the test tube from the bath, stir gently with a glass rod, and cool at room temperature for about 2-3 minutes. At this time, the agarose solution should be absolutely clear.

* The melted agarose for the four gels (15ml per gel) can also be prepared in one operation by boiling 0.9g of agarose in 75ml of buffer in a 250-500ml flask over a bunsen burner or in a microwave oven. The flask should be rotated periodically during the heating process in order to prevent damage to the agarose.

3. Using the same pipet, transfer the contents of this tube back into the larger vial that contains most of the CaCl_2 solution.

4. Tap the vial with the tip of your index finger to mix the solution.

5. Incubate the cells for about 20 minutes on ice. The cells are then called competent because they can take up DNA from the medium. If desired, the cells can be stored in the CaCl_2 solution for up to 12 hours on ice before use.

II. Uptake of DNA by competent cells

1. Choosing plasmid samples.

The analysis described below was designed for 8 groups of students. There are three tubes labeled Plasmid (A, B, and C). Each group will analyze two plasmids as indicated below.

<u>Student Group Number</u>	<u>Plasmids that will be analyzed</u>
1, 2 -	Plasmids A and B
3, 4 -	Plasmid A and B
5, 6 -	Plasmid A and C
7, 8 -	Plasmid B and C

2. Obtain two sterile tubes (1.5ml) and label the tubes with the letters that correspond to the plasmids that you will analyze (A and B, A and C, or B and C)

3. Using a sterile micropipet, add 10 μl of the corresponding plasmid DNAs to the two tubes and place them in an ice bath. Use a fresh sterile micropipet for each addition.

4. Gently tap the vial of competent cells with the tip of your index finger to ensure that the cells are in suspension. Then, using a sterile transfer pipet, add 5 drops (5 drops $\approx 100 \mu\text{l}$) of the competent cells to each of the two tubes. Tap each of these tubes with the tip of your index finger to mix these solutions and store both tubes on ice for 30 - 90 minutes. During this time, proceed to Part B of this exercise: Analysis of Plasmid Genotype. Return to step 5 (below) during the electrophoretic run.

5. Transfer the tubes to a water bath, preheated to 37°C, for 5 minutes. This heat shock facilitates the uptake of plasmid DNA.

Electrophoresis

1. Transfer the four casting trays with gels to the central platform of the electrophoresis cell and position them such that the sample wells are closest to the black (negative) electrode. Upon electrophoresis, most proteins will then migrate from the negative (black) towards the positive (red) electrode.
2. Place the gel tray stabilizing bar parallel to the long axis of the electrophoresis cell between the gel trays.
3. Slowly fill the electrophoresis chamber with electrophoresis buffer until the four gels are covered with a 1/4 cm layer of buffer. Approximately 2.5 liters of buffer are required.
4. Place the electrophoresis cell lid in position.
5. With the power supply off, connect the cables from the cell to the power supply, red to red (positive) and black to black (negative).
6. Push the rocker switches on the power supply to "on" and "170V". The voltage will now remain constant at 170 volts during the run.
7. Unless otherwise indicated, electrophorese until the bromophenol blue in the sample solution has migrated to within 1/4 cm of the positive electrode end of the gel. At 170 V, this takes approximately 50 minutes.
8. At the termination of the electrophoretic separation, shut off the power supply, disconnect the cables and remove the gel casting trays containing the gels.
9. The buffer should be emptied from the electrophoresis cell and stored under refrigeration in a separate container until the next electrophoretic run. The same buffer should be used for at least 3 electrophoretic separations. However, fresh buffer should be employed for the preparation of all agarose gels. The electrophoresis cell should be rinsed with water and stored in an upright position.

- D. Think about what you are going to do before you do it. Common sense is the best defense against laboratory accidents and contamination.

In this exercise, plasmids will be introduced into *E. coli* by transformation. There are three basic steps to the procedure: I) Treating bacterial cells with calcium chloride in order to enhance the uptake of plasmid DNA. II) Incubating the competent cells with plasmid DNA. III) Selection of those cells that have taken up the plasmid DNA by growth on an ampicillin-containing medium.

Step I should be performed by the instructor before or at the beginning of the laboratory session. Eight students or eight teams of students should then perform steps II and III.

1. Preparation of Competent Cells

(This step should be performed by the instructor).

1. Place the vial of CaCl_2 and the tube of *E. coli* in the ice bath.

2. Using a sterile pipet, transfer about 1/2 ml of the CaCl_2 solution to the tube containing the *E. coli* culture.

I. Preparation of Competent Cells

- Step I** should be performed by the instructor before or at the beginning of the laboratory session. Eight students or eight teams of students should perform steps II and III.

In this exercise, plasmids will be introduced into E. coli competent cells with plasmid DNA and the uptake of plasmid DNA by growth on an ampicillin-containing medium.

D. Think about what you are going to do before you go to the defense against laboratory ad-

C. Place used cultures and contaminated needles in
tacles. These should be sterilized in
concentrated bleach prior to disposal.

objects on the lab bench.

B. Expose open petri dishes and tubes with media to the open air as little as possible, never mouth pipet culture solutions, and never place sterile

A. Wash your hands before and after completion of the exercise, and several times for working with bacteria in the laboratory.

1. Prior to performing this exercise, the student should review the following:

Procedure

Part A. Analyses of the *E. coli* Phenotype after Transformation

Electrophoresis. The procedure is written such that both parts can be performed during a single 3-hour laboratory session. Alternatively, parts A and B can be performed independently during two 2-hour laboratory periods.

2. Bollag, Daniel M. Protein Methods New York: Wiley - Liss, 1991.

2. Albers B., Bray, D., Lewis, J., Raff, M., Roberts, K., Wilson, J. D. Molecular Biology of the Cell. Garland Publishing, Inc. New York and London (1994).

1. Gel Electrophoresis of Proteins: A Practical Approach, Oxford, New York: IRL Press (1992).

1. Mosher, R. A. The Dynamics of Electrophoresis. Weinheim; New York: VCH (1992).

ELECTROPHORESIS.

2. Albers B., Bray, D., Lewis, J., Raft, M., Roberts, K., Watson, J., D. Molecular Biology of the Cell. Garland Publishing, Inc. New York and London (1994).

PROJEKT BIOCHEMISTRE

- #### V. SUGGESTED READING AND REFERENCES FOR PART A

Chemical and Desulfurizing

- Safety gloves should be used during the staining and destaining procedures in order to avoid contact with acidic acid, methanol, and stain.

the lac Z gene which is the gene for β -galactosidase. The plasmid preparations will be characterized in two ways. Plasmid genotype will be studied by restriction nuclease digestion and electrophoresis. The phenotype of *E. coli* will also be examined after introduction of the plasmids into the bacteria by transformation. The cells will be plated onto nutrient agar containing ampicillin and Xgal. Analysis of bacterial colony growth and color will enable you to determine if the plasmid contains functionally active genes for ampicillin resistance and β -galactosidase. The results of the genotypic and phenotypic studies will permit you to determine the nature of the plasmids in tubes A, B, and C.

Materials Provided

*Plasmid A
*Plasmid B
*Plasmid C | Note: one tube does not contain plasmid: one tube contains plasmid pUC18, and one tube contains a recombinant plasmid with the foreign DNA inserted into the EcoRI site located in the gene for β -galactosidase

DNA markers (for electrophoresis)

**EcoRI Endonuclease (contains EcoRI dissolved in restriction nuclease buffer)

Sample Buffer (for electrophoresis)**X-gal - Ampicillin - Agar plates (16)

Nutrient Broth (10ml) - Store at 4°C.

25 Inoculating loops

19 large sterile Transfer pipets - One pipet should be used for the bacteria, one for the nutrient broth and 16 for step III.

16 Sterile tubes

CaCl₂ solution (5 ml) - 100 mM CaCl₂

E. coli (0.5ml) - Store at -20 to -70°C

Materials Not Provided

Water bath maintained at 37°C - If a temperature regulated water bath is not available, a beaker containing tap water at 37°C (98°F) will suffice.

Ice bath - Ice chips in a large beaker are suitable.

Air Incubator maintained at 37°C (OPTIONAL - see below).

Agarose, Electrophoresis Buffer, and Gel Stain

**Sterile microliter pipets

* Identified in the Instructor Guide

**Prepared as described in the Instructor Guide

Laboratory Schedule

The exercise described below is divided into two parts: Part A. Analysis of *E. coli* Phenotype after Transformation; Part B. Analysis of Plasmid Genotype

PART B

Experiment 1 (101). Electrophoretic Separation of Proteins. Background Information

The isoelectric point of a protein is defined as the pH at which the protein does not migrate in an electric field (see Section II). The isoelectric points of the four colored proteins that you will study in this exercise are listed in Table 1-1. A brief description of the functions and properties of these proteins is given below.

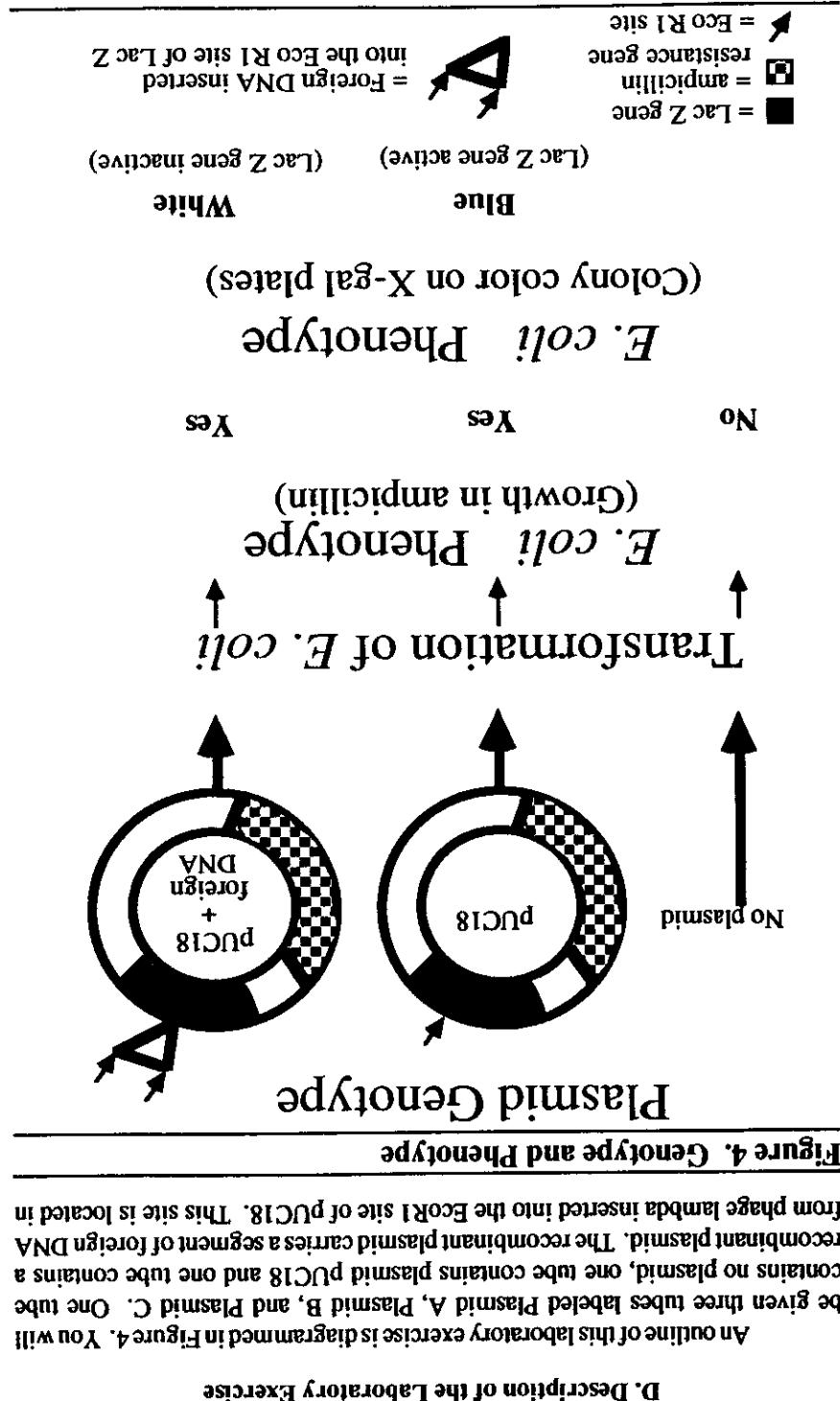
Table 1-1. Properties of the Proteins Used in this Exercise

Protein	Color	Isoelectric Point	Net Change at pH 8.6
Cytochrome C	Orange	10.2	Positive
Myoglobin	Brown-red	7.2	Negative
Hemoglobin	Red	6.8	Negative
Serum Albumin*	Blue	4.8	Very negative

*Bromophenol blue has been added to the serum albumin sample which serves to stain the protein blue.

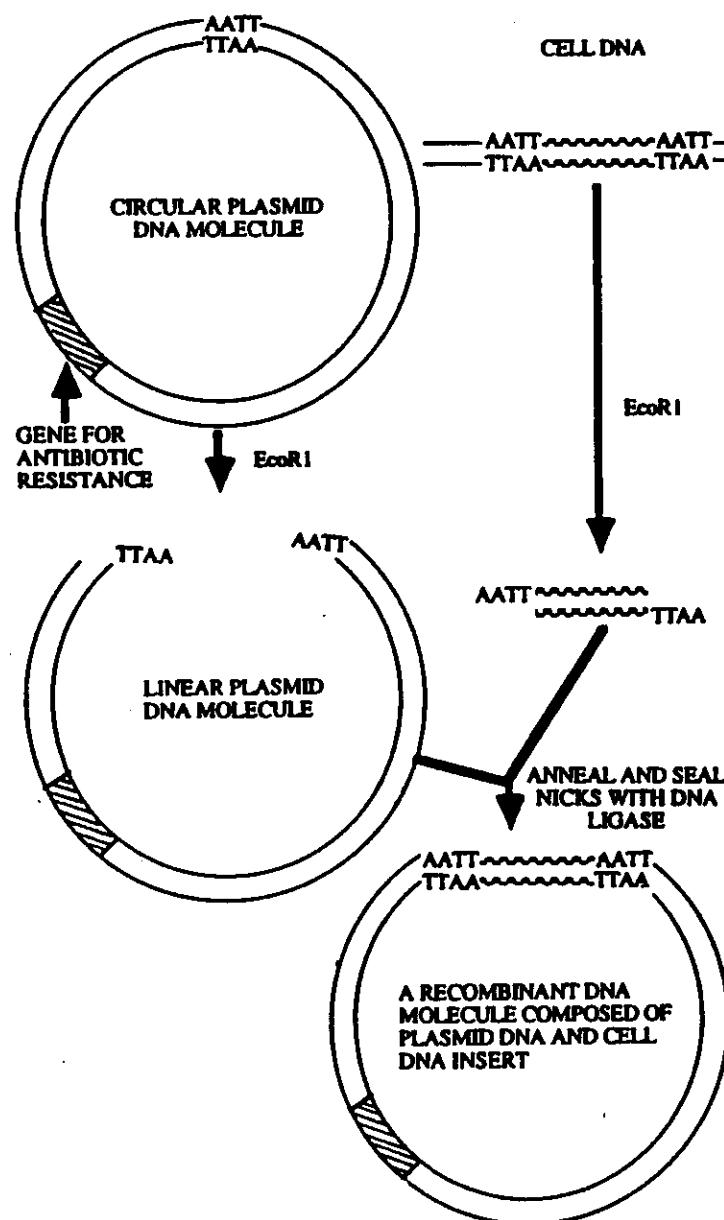
Cytochrome C - Plant and animal tissues contain a class of cell protein pigments called cytochromes. Cytochrome C, which is one of the most well characterized of the cytochromes, is an integral part of the electron transport system in mitochondria and is involved in cell energy production. Cytochrome C consists of a single polypeptide chain which is wound around a central, nonproteinaceous compound called heme. It is the iron containing heme group which is responsible for the orange-brown color of this protein. The protein is basic in nature primarily because it contains a high concentration of lysine residues. The isoelectric point of horse cytochrome C is 10.2 and at pH 8.6 the protein carries a net positive charge. Thus cytochrome C, unlike most proteins, migrates to the negative electrode during electrophoresis at pH 8.6.

Myoglobin - Myoglobin and hemoglobin also contain an iron containing heme group and the iron is involved in oxygen binding. Myoglobin binds and stores oxygen in muscle and hemoglobin is involved in the transport of oxygen in blood. The isoelectric point of myoglobin from horse is 7.2. Thus, this protein should move toward the positive electrode during the electrophoretic separation.



- Materials**
- I. The solutions and materials required for electrophoresis, sample handling and gel staining and destaining (see Instructor Guide).
- II. The four proteins listed below are provided in the Chemical Package.
- Cytochrome C - The protein is from horse heart.
 - Myoglobin - This protein was isolated from cow muscle.
 - Hemoglobin - The hemoglobin is from rabbit red cells.
 - Serum albumin - The albumin is from cow.
- Procedure**
- The procedures for the preparation, electrophoresis, and staining of the agarose gels are described in detail in Section IV (pages 13-16) of this manual and briefly outlined below. This experiment was designed such that the samples of agarose gels are described in detail in Section IV (pages 13-16) of this manual and students will share one gel. The experiments of each student (or student pair) will be電気泳動分析 on four gel lanes.
- I. Prepare the agarose gel.
- Two students will be assigned one agarose gel. If the students work in pairs, four students will share one gel. The experiments of each student (or student pair) will be電気泳動分析 on four gel lanes.

Figure 3. Preparation of a Recombinant DNA Molecule



2. Load 15 μ l of each of the four colored proteins into the sample wells as indicated below.

<u>Sample Well Number</u>	<u>Protein Sample</u>
1	Cytochrome C
2	Myoglobin
3	Hemoglobin
4	Serum Albumin
5	Cytochrome C
6	Myoglobin
7	Hemoglobin
8	Serum Albumin

- Transfer your gel to the electrophoretic cell making sure you note the position of your gel in the electrophoresis chamber.
- Electrophorese for 8 minutes, and then turn off and disconnect the power supply.
- Remove the lid of the electrophoresis unit and note the relative position of the four proteins as compared to their point of application at the sample wells.
- Resume electrophoresis until the bromophenol blue in the serum albumin sample has migrated to within 1 cm of the positive electrode end of the gel. Remove the gels from the unit and measure the distance of the proteins (in cm) from the sample wells. For long term storage of the gels, place them in destain solution for at least 1 hour and then perform step 6 on page 16.
- (Optional) Stain and destain the agarose gels.
- Measure the distance of the proteins (in cm) from the sample origin.

Study Questions

- What is the molecular basis for the differences in the electrophoretic mobilities of the four proteins analyzed in this exercise? Relate your answer to the isoelectric points of these proteins that are given in Table 1-1.
- Explain how your results would have been affected if the electrophoretic separation was carried out at pH 10.5.
- Explain how your results would have been affected if the separation was carried out at pH 6.0.

Plasmids are useful tools for the molecular biologist because they serve as gene-carrier molecules. A basic procedure of recombinant DNA technology consists of joining a gene of interest to plasmid DNA to form a hybrid, or recombinant molecule that is able to replicate in bacteria (Figure 3). In order to prepare a recombinant molecule, the plasmid and gene of interest are cut at precise positions by restriction endonucleases and then the molecules are spliced together using an enzyme called DNA ligase. After the hybrid molecule has been prepared, it is introduced into E. coli cells by transfection. The hybrid plasmid purified from the bacterial cells to produce an enormous number of copies replicates in the dividing bacterial cells to produce large quantities of more interest is recovered. This method has enabled scientists to obtain large quantities of more than 1000 specific genes from the genome of the original gene. At the end of the growth period, the hybrid molecules are purified from the bacteria and the original gene.

C. Plasmids as Gene Carrier Molecules

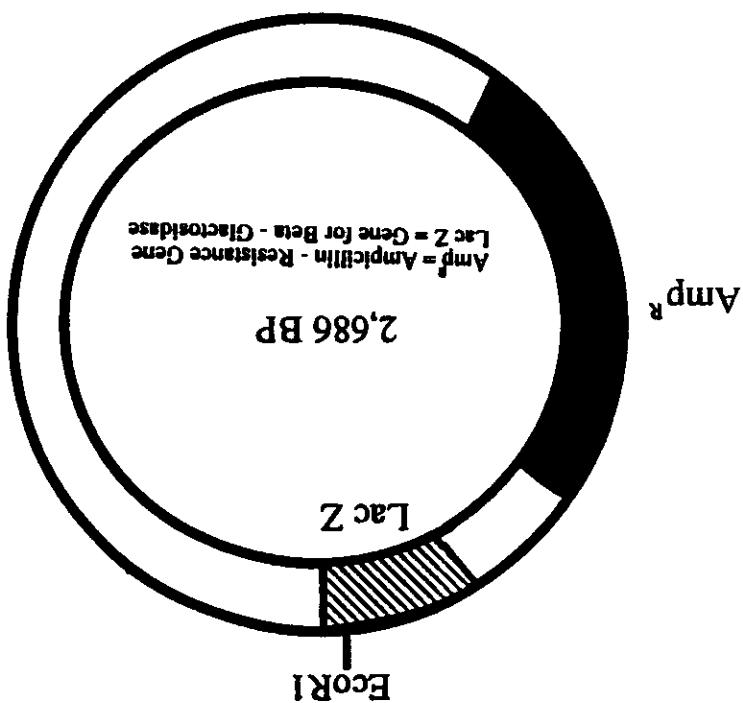


Figure 2. Partial Map of Plasmid pUC18.

The gene for hemoglobin S is thought to have originated in the Black population in Africa. Hemoglobin S has an important benefit in the African population because it confers resistance to one form of malaria. In some parts of Africa, 40% of the population have the sickle cell trait. The incidence of sickle cell

Sickle cell disease is inherited as an autosomal recessive gene. When the gene for hemoglobin S is inherited from only one parent, the individual is heterozygous for hemoglobin S and has sickle cell trait. Although these individuals rarely have severe anemia, half of their circulating hemoglobin is hemoglobin S and half is normal hemoglobin A. When the gene for hemoglobin S is inherited from both parents, the individual is homozygous. In this case, all of the hemoglobin is hemoglobin S and the individual suffers from severe anemia.

Many changes in the structure of hemoglobin have arisen by mutations in the human population. About one person in 100 contains a mutant hemoglobin gene and these individuals have an abnormal hemoglobin molecule in their blood. The mutations often involve substitution of one amino acid for another and usually they are harmless. However, in a few cases, mutations in hemoglobin can cause serious diseases. One of the most common and serious abnormalities in hemoglobin is hemoglobin S, which is present in individuals suffering from sickle cell anemia. In hemoglobin S, a single glutamic acid residue on the β -chains is replaced by valine. This single change in the primary sequence causes a marked change in the net charge and conformation of the protein. When hemoglobin S is deoxygenated, it crystallizes in the red blood cells and this leads to a distortion of the red cells into a sickle shape. These abnormal cells are then destroyed rapidly in the body which leads to a reduced number of erythrocytes; hence the term, sickle cell anemia.

Red blood cells, or erythrocytes, carry the protein hemoglobin in the circulatory system. This protein serves to transport oxygen from the lungs to the tissue. Hemoglobin is a globular protein made up of 4 subunits. Each subunit contains a polypeptide chain attached to an iron-containing component called heme. Each heme group contains one iron atom and each iron atom can bind one O₂ molecule. The polypeptide chains of hemoglobin are referred to as the globin portion of the molecule. Normal adult hemoglobin (hemoglobin A) has four sub-units made up of two alpha (α) and two beta (β) polypeptide chains. Each chain contains 141 amino acid residues and each β-chain contains 146 amino acid residues. Each α-chain contains 146 amino acid residues and each β-chain contains 141 amino acid residues. Hemoglobin F is similar to hemoglobin A except that gamma (γ) chains replace the β-chains. Although adult hemoglobin normally replaces fetal hemoglobin shortly after birth, fetal hemoglobin is found in the blood of the human fetus. Hemoglobin F is similar to hemoglobin F is found in the blood of the human fetus. Hemoglobin F is similar to hemoglobin A except that gamma (γ) chains replace the β-chains. Although adult hemoglobin normally replaces fetal hemoglobin shortly after birth, fetal hemoglobin is found in the blood of the human fetus.

Background Information

Experiment 2 (102). Sickle Cell Anemia

Restriction Sites

To be useful as a cloning vector, a plasmid should possess a single recognition site for one or more restriction enzymes into which foreign DNA can be inserted. One way to increase the number of such restriction sites is to add a polylinker, a segment of synthetic DNA that contains closely spaced restriction sites. Plasmid pUC18 carries a 54 base-pair polylinker that contains unique sites for 13 different restriction enzymes. Note that the polylinker contains a unique site for EcoR1 which will be exploited in the analysis described below.

Replication

Replication of plasmid DNA requires a plasmid replication origin and a number of replication enzymes. The replication enzymes are usually provided by the host bacterial cell. The replication of some plasmids is coupled to that of the host cell. As a result of this so-called "stringent control," only one or at most a few copies of the plasmid will be present in each bacterial cell. In plasmids under "relaxed control," replication of the plasmid DNA is not coupled to replication of the host DNA chromosome and more than 500 copies of the plasmid may be present in a single host cell. Plasmid pUC18, as well as other common plasmid-cloning vectors, are under relaxed control.

Lac Z gene

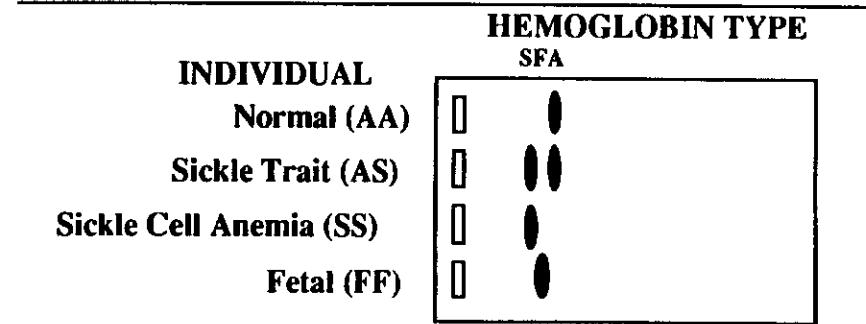
β -Galactosidase is an enzyme which hydrolyzes lactose and other β -galactosides into component sugars. The activity of the enzyme is usually measured with chromogenic substrates which, when hydrolyzed, produce colored products. One substrate is Xgal (5-bromo-4-chloro-3-indolyl- β D galactoside) which is converted to a blue product by β -galactosidase. Xgal is frequently used to detect β -galactosidase in cell extracts, bacterial colonies, and phage plaques. You will use this substrate to identify *E. coli* colonies containing β -galactosidase.

The Lac Z gene, which specifies β -galactosidase, is a member of the *E. coli* Lac operon. Plasmid pUC18 contains a portion of the *E. coli* Lac Z gene that codes for the first 146 amino acids of β -galactosidase. The remaining portion of the gene is encoded by appropriate strains of *E. coli* such as DH5 and JM 101. Thus, when these strains are transformed with pUC18, complementation occurs and the bacteria produce active β -galactosidase, which gives rise to blue colonies on nutrient agar containing Xgal.

The above complementation forms the basis of a powerful selection method that you will use to identify *E. coli* colonies containing pUC18 with foreign DNA. The polylinker is contained within the Lac Z gene of pUC18 although the insertion does not affect the ability of the β -galactosidase peptide to be complemented by the *E. coli* host. However, foreign DNA inserted into the polylinker destroys complementation and active β -galactosidase is not synthesized. Thus, in the later case, white colonies are produced on Xgal plates in contrast to the blue colonies produced by cells containing pUC18 without foreign DNA.

trait in the American Black population is about 10%, and about 1-2% of this population has sickle cell anemia.

Abnormal hemoglobins are often detected in the clinical laboratory by electrophoresis. Hemoglobin molecules at pH 9.2 have a net negative charge and move toward the anode in an electrophoretic system at a speed proportional to the strength of their charge. The isoelectric point of normal hemoglobin A is 6.9 but the isoelectric point of certain hemoglobin variants differ depending on the hemoglobin type. As discussed above, valine residues replace the glutamic acid residues in the β chains of hemoglobin S. Thus, hemoglobin S has two fewer negative charges per hemoglobin molecule than hemoglobin A and its isoelectric point and electrophoretic properties are correspondingly affected. Hemoglobin variants including sickle cell, sickle trait, persistence of fetal hemoglobin in the adult, and many others can be detected by electrophoresis. The Figure shown below illustrates the electrophoretic separation of the normal hemoglobin and some common hemoglobin variants.



Objectives In this experiment, you will examine the electrophoretic behavior of hemoglobin from a normal person and from a person with sickle cell trait. In addition, an optional part of this experiment is for the student to examine his or her own hemoglobin.

Materials

- I. The solutions and materials required for electrophoresis, sample handling and gel staining and destaining (see Appendix 1 and 2 - instructor manual). Note that the hemoglobin electrophoresis buffer is to be used for this experiment. The pH of this buffer is 9.2.
- II. The following solutions are provided :
 1. Normal human hemoglobin
 2. Sickle trait hemoglobin
 3. Sickling hemoglobin
 4. Erythrocyte lysis buffer

The following materials are for the optional part of this exercise and are not provided in the Chemical Package.

Figure 1. Transformation of Bacteria with Plasmid DNA

The diagram illustrates the transformation of bacteria with plasmid DNA. It starts with an 'Antibiotic Sensitive Cell' containing 'Bacterial DNA'. An arrow labeled 'Calcium Chloride' points to the cell, followed by an arrow labeled 'Competent Cell'. This leads to a 'Transformed Antibiotic Resistant Cell' containing both 'Bacterial DNA' and 'Plasmid with Antibiotic Resistance Gene'. An arrow labeled 'Erythrocyte Lysis Buffer' points to the cell, followed by an arrow labeled 'Cells'. This results in a 'Colony of Antibiotic Resistant Cells'.

B. Plasmid PUC18

The plasmid PUC18 is shown in Figure 2 and a discussion of some of the features of this plasmid that you will use in the analysis is called PUC18. A map of plasmid PUC18 is given below.

Plasmid PUC18 contains an ampicillin-resistance gene that enables E. coli to grow in the presence of the antibiotic. Thus, bacteria containing this plasmid will lose the plasmid, grow in the absence of this antibiotic, and other enzymes of this antibiotic. The ampicillin-resistance gene of PUC18 codes for the enzyme beta-lactamase (penicillinase), which inactivates ampicillin and other penicillins.

Like all plasmids, PUC18 is a circular DNA molecule. Digestion of the plasmid with a restriction endonuclease that cleaves the DNA in only one site converts the circular form to a linear molecule. Digestion of the plasmid with a restriction endonuclease that makes it less susceptible to physical damage = 2×10^9 . The small size of this plasmid makes it less susceptible to physical damage than handling. In addition, smaller plasmids generally replicate more efficiently in bacteria and produce larger numbers of plasmids per cell.

*If student hemoglobin samples are not available, electrophoresis standard normal and sickle trait hemoglobin on lanes 4 and 8.

Sample Well Number	Protein Sample*
1	Normal hemoglobin - Hemoglobin AA
2	Sickle trait hemoglobin - Hemoglobin AS
3	Sickle trait hemoglobin - Hemoglobin SS
4	Student hemoglobin
5	Normal hemoglobin standard - Hemoglobin AA
6	Sickle trait hemoglobin - Hemoglobin AS
7	Sickle trait hemoglobin - Hemoglobin SS
8	Student hemoglobin

1. Prepare 1.2% agarose gels and load 15 µl of the following hemoglobin solution into the sample wells.
- II. Electrophoresis

3. Remove 20 µl of the blood and add it to 0.1 ml (about 3-4 drops) of the erythrocyte lysis buffer. Mix the contents of the tube thoroughly. The erythrocyte lysis buffer contains the detergent Nonidet P-40 which serves to lyse (break open) the erythrocytes which in turn liberate their hemoglobin. Although the final solution contains plasma proteins as well as hemoglobin, the latter is a major component of the mixture and can readily be detected after electrophoresis.
2. Allow 1 drop of blood to fall into a small test tube.

1. Disinfect your finger with 70% alcohol, allow it to dry and puncture it with a sterile finger lancet.
- I. Preparation of the Hemoglobin Sample (Optional)

3. Sterile nonabsorbent cotton
2. 70% alcohol
1. Sterile finger lancets
- Procedure

The following materials are for the optional part of this exercise and are not provided in the Chemical Package.

Experiment 1004. Genotype to Phenotype

Background Information

A. Introducing a Plasmid into *E. coli*

Penicillin is one of the most important anti-infective agents used in clinical medicine because it is inexpensive, bactericidal and its toxicity for human cells is almost nonexistent. Penicillin interferes with the synthesis of the bacterial cell wall and will thus cause osmotic lysis of susceptible microbes. Penicillin is not a single compound but a group of compounds with related structures and activities. Many of these compounds are semi-synthetic in that part of each molecule is made by a mold to which the chemist adds another chemical group. Over 500 semi-synthetic penicillins have been made during the past 30 years. Ampicillin is a broad-spectrum semi-synthetic penicillin that will kill a number of gram-positive and gram-negative bacteria, including *Salmonella* and *Escherichia coli*. Occasionally, *E. coli* cells are found in nature that are resistant to the toxic effects of ampicillin. In today's laboratory, you will create such an ampicillin-resistant population of *E. coli*.

Plasmids are small circular DNA molecules that exist apart from the chromosomes in most bacterial species. Under normal circumstances, plasmids are not essential for survival of the host bacteria. However, many plasmids contain genes that enable bacteria to survive and to prosper in certain environments. For example, some plasmids carry one or more genes that confer resistance to antibiotics. A bacterial cell containing such a plasmid can live and multiply in the presence of the drug. Indeed, antibiotic-resistant *E. coli* isolated in many parts of the world contain plasmids that carry the genetic information for protein products that interfere with the action of many different antibiotics. In this laboratory, you will introduce a plasmid that contains an ampicillin resistance gene into *E. coli*.

In the laboratory, plasmids can be introduced into living bacterial cells by a process known as transformation. A common procedure for transformation is shown in Figure 1 and entails:

1. The treatment of bacterial cells with calcium chloride in order to enhance the uptake of plasmid DNA. Such calcium chloride treated cells are said to be competent.
2. The incubation of the competent cells with plasmid DNA and the uptake of the DNA into a small fraction of the cells. The cells that have taken up plasmid DNA are said to be transformed.
3. The selection of transformed cells by growth on an antibiotic-containing medium.

2. Electrophorese until the bromophenol blue in the samples has migrated to within 1/4 cm of the positive electrode end of the gel.
3. Since hemoglobin has its own inherent color, it can sometimes be visualized on the gels without staining. Inspect the gels carefully to see if you can identify the hemoglobin bands.
4. Stain and destain the gels and record your results noting the migration of hemoglobin A, hemoglobin S and the hemoglobins from yourself and your classmates.

Study Questions

1. What is the molecular basis for the difference in the electrophoretic pattern between normal hemoglobin A and hemoglobin S?
2. Can all abnormal hemoglobins be diagnosed by electrophoresis? Explain your answer in detail.
3. What fraction of the offspring of two parents with sickle trait would you expect to have sickle cell anemia?

Experiment I (801). Identification and Evolutionary Relationship of Serum Proteins by the Western Press-Blot Procedure

dye by rinsing the slides in water and then permit the slides to air dry for at least 10 minutes before microscopic examination. The slides can be viewed at this time or stored until the next laboratory session.

6. Locate the cells and nuclei under the low power of your microscope and then add a drop of oil and examine them with the oil immersion lens. The nuclei should stain purple and the cytoplasm pale blue. L�enally the plasma membrane, cytoplasm and nuclei on slide A and compare the nuclei on slide A to those on slide B. Also note the nucleoli, which will appear as small dark blue dots in the nuclei.

1. Compare the morphology of the isolated nuclei to the nuclei in intact thymus cells.
2. Describe the changes in the clarity and viscosity of the nuclear suspension that occurred upon addition of SDS. Why did these changes occur?
3. Thymus cells have a relatively small amount of cytoplasm and this feature makes thymus tissue ideal for DNA extraction. Why?

Each protein carries in its amino acid sequence information pertaining to its own evolutionary history and origin, and clues to the evolutionary history of the species in which it is found. Indeed, the millions of proteins existing today are, in effect, living fossils. Two of the methods that have been used to study the evolution of proteins at the molecular level are described below.

The theory of organic evolution is based upon the belief that present-day organisms have descended with modifications from forms of life that lived in the past. Phylogeny is the evolutionary history of a species, genus or larger group and taxonomy is the science of classification of organisms according to the degree of kinship and evolutionary relationships. The doctrine of organic evolution and comparative paleontology and geobiography have provided strong evidence for the doctrine of organic evolution and have also provided strong evidence for the modern biocultural laboratory have anatomy and embryology. Results from the modern biocultural laboratory have from genetics, paleontology and geobiography and from comparative drawn the most important generalizations in science. It is supported by evidence drawn from most probable mechanisms by which evolutionary changes occur.

A comparison of the amino acid sequence of the same protein in different organisms has provided a direct way to study molecular evolution. A comparison of the amino acid sequence of cytochrome C from over 80 species has revealed that the amino acid sequences of this protein from different species and the degree of variation corresponds to the distance that separates two species on the evolutionary tree. That is, the greater the taxonomic difference, the more the cytochromes are likely to differ in their order of amino acid residues. For example, the cytochromes C in molecules in men and chimpanzees contain 104 amino acid residues and the order of their amino acid residues is exactly the same. In contrast, the cytochrome C in man differs from the cytochrome C found in yeast in 44 out of the 104 amino acid residues. The numbers of amino acid replacements in cytochrome C of 12 species are compared in the table below. This type of information has led to the conclusion of family trees of organisms that agree remarkably well with those obtained from comparative protein studies (See Figure 1-1). In fact, on a number of occasions, the classical anatomical record (See Figure 1-1) has been used to clarify and expand on phylogenetic relationships that were derived from classical analysis.

Background Information

I. PROTEIN SEQUENCE

2. Using a large transfer pipet, add 1ml of the SDS solution to the vial and mix the contents of the vial carefully. Note and record below the appearance and consistency of this solution.

Appearance: _____

Consistency: _____

3. After 5 minutes, carefully pour the cold alcohol into the vial so that the alcohol forms a layer on top of the solution. Add enough alcohol so that the vial is nearly full. Dip one end of the rod into the vial through the cold alcohol, into the DNA. Slowly rotate the rod and then raise it back into the alcohol. Fine DNA fibers should form on the end of the rod. Produce more of these DNA fibers by turning the rod and moving it up and down between the DNA and the alcohol.
4. After no more fibers can be formed, remove the rod and examine the DNA fibers wound around it. The fibers are the alcohol-insoluble form of DNA, contaminated with some proteins.

III. Microscopic Analysis

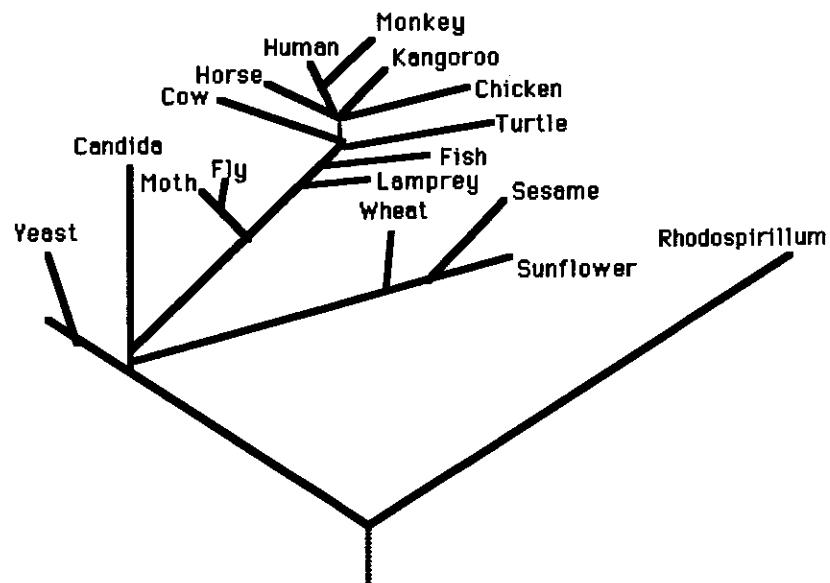
In this section, you will compare the nuclei in intact thymus cells to the nuclei that were isolated in Section I.

1. Obtain two clean microscope slides from your instructor and label one slide A and one slide B.
2. Using the blunt end of a toothpick, scrape the surface of one of the sections of thymus from Section I to remove a few cells. Smear these cells onto the center of the slide labeled A and allow the preparation to dry.
3. Using a transfer pipet, apply a small drop of the nuclear suspension to the center of the slide labeled B. Smear the nuclei on the slide using a clean toothpick and allow the preparation to dry.
4. In general, cells and organelles must be fixed before they can be stained. Fixation makes cells permeable to stains and causes them to adhere to the surface of the slide so they will not be washed away during the staining procedures. Place three drops of alcohol onto the slides in order to fix the preparations. After two minutes, rinse the slides in water.
5. Cells and cell organelles are almost invisible by standard light microscopy unless they are stained. To stain your preparations, place 3 drops of the nuclear stain onto your slides and stain for 5-10 minutes. Remove excess

Variations in the Amino Acid Sequence of Cytochrome C

Cytochromes compared to human cytochrome	Number of variant amino acid residues
Chimpanzee	0
Rhesus monkey	1
Kangaroo	10
Dog	11
Horse	12
Chicken	13
Rattlesnake	14
Tuna fish	21
Dogfish	23
Moth	31
Wheat	35
Neurospora	43
Yeast	44

Figure 1-1. A Family Tree of Organisms Based on the Amino Acid Sequence of Cytochrome C.



- Consistency: Apperance:

of this suspension.

1. One member from each group should place 2ml of the nuclear suspension into a glass vial. Note and record below the appearance and consistency of this suspension.

These proteins bind to DNA.

These giant molecules bind chromatin into compacted chromatin. In order to liberate the DNA from chromatin, these proteins must be removed. One method to dissociate the histones from DNA uses the protein de-naturant sodium dodecyl sulfate (SDS). This de-naturant alters the histones, thereby disrupting the non-covalent bonds that link these giant molecules into compacted chromatin.

2. Place a few of the tissue sections into a pre-cooled beaker. These sections will be used for the microscopic analysis described in Section III.

3. To the remainder of the tissue, add 70ml of cold nuclear buffer.

4. Pour the tissue-buffer mixture into a chilled blender and add about 10cm³ of ice chips. Turn on the blender and homogenize for 60 seconds. The mechanical action of the blender as well as the chemical action of a detergent that is present in the nuclear buffer should disrupt the plasma membrane leaving the nuclear envelope intact. If a blender is not available, pour the tissue-buffer mixture into a pre-cooled mortar in small portions and grind the tissue sections with the pestle until a homogeneous suspension is formed.

5. Filter the homogeneous through 2 layers of cheese cloth and discard the suspension.

6. Carefully pour off and discard the supernatant (top liquid layer). The pellets at the bottom of the centrifuge tubes contain nuclei.

7. Transfer the nuclear pellets to the chilled blender and add 70ml of cold nuclear buffer.

8. Blend the pellets for 10 seconds, filter the nuclear suspension through 2 layers of cheese cloth and store it in a chilled state.

1. DNA Isolation

III. IMMUNOLOGICAL PROCEDURES

The macromolecules that elicit antibody production are called antigens and most often proteinaceous in nature. Although antigens are frequently composed of foreign organisms, purified foreign proteins will serve as antigens in that they will stimulate the formation of antibodies when injected into a suitable test animal such as rabbit. Each antigen possesses features that are recognized by the immune system of about 5 to 10 amino acid residues on the protein molecule. It follows that each protein possesses a large number of potential antigenic determinants and a sequence of about 5 to 10 amino acid residues on the protein molecule. It follows that each protein recognizes the antigenic determinants or epitopes. An antibody and these features constitute the unique shape of an antigenic determinant. These features consist of the antigenic determinants along the protein.

Antibodies are frequently used to study evolutionary relationships because they recognize unique antigenic determinants along a protein. For example, human serum albumin infected into a rabbit molecule. For rabbit serum albumin antibodies are produced in the serum. These antibodies are directed against determinants of human albumin (shapes and/or amino acid sequence) that are not found on the human albumin. Similarly, the antibodies are directed against determinants of rabbit albumin. The antibodies in the serum react strongly with the rabbit albumin molecule. When the anti-albumin serum is mixed with human albumin antisera will react only weakly with albumin from deer, horse and ox and not at all with chicken or frog albumin.

In this experiment, you will observe the evolution of antibodies in sera from various vertebrates. First, you will observe the albumin and gamma globulins from various vertebrates. Then, you will compare the patterns of electrophoretically separated proteins present in sera from cow, goat, sheep, horse, and chicken. You will then perform an immunological procedure known as Western Blotting. Through a series of steps (described below), this procedure enables the investigator to visualize proteins that react with a specific antibody as Western Blotting. Thus, the proteins have been separated by electrophoresis. This antisera will react with albumin from goat, sheep, horse, and chicken. You will then observe the evolution of antibodies in sera from various vertebrates. Finally, you will compare the patterns of electrophoretically separated proteins present in sera from cow, goat, sheep, horse, and chicken. This antisera will react with albumin from deer, horse and ox and not at all with chicken or frog albumin.

The immune system consists of a diverse set of cells, tissues and organs and about 10^{12} antibody molecules. The major function of the immune system is to protect the organism from viruses, bacteria, protozoans and larger parasites. When these organisms enter the vertebrate body, macromolecules on their surfaces induce the production of specific antibodies that appear in the serum of the infected animal. The antibodies, in turn, combine with these foreign macromolecules thereby rendering the invading organisms inactive and noninfective. Central to this protective mechanism is the ability of the immune system to distinguish between foreign macromolecules and those that are a natural part of the individual's body.

Objective To isolate DNA from the nuclei of eukaryotic cells.

Materials Provided

Calf Thymus (15g) - The thymus should be thawed at the beginning of the class period by placing the bag containing the tissue in warm water.

8 glass vials

8 rods for DNA spooling

Transfer pipets

Sodium Dodecyl Sulfate (SDS)

*Nuclear stain

Cheese cloth

*Nuclear buffer - This buffer should be placed in the refrigerator at least 3 hours before the laboratory period.

*Prepared as described in the Instructor's Guide

Materials Not Provided

Ethyl alcohol - The alcohol should be placed in a freezer at least 3 hours before the laboratory period. (See Instructor Guide)

Ice bath - ice chips in a large beaker are suitable

Microscope slides (16) and microscopes

Centrifuge and centrifuge tubes (10ml-30ml capacity) - Recommended but not absolutely necessary. A small tabletop clinical centrifuge is suitable for this exercise.

A device for tissue disruption such as a food blender (Waring or similar types), or a mortar with pestle. The device should be precooled before the laboratory session.

Funnel

Beakers

Toothpicks

Scissors

Procedure

There are three basic parts to this exercise: (I) Isolating nuclei from calf thymus cells. (II) Preparing DNA from these nuclei and (III) Comparing the isolated nuclei to the nuclei in intact thymus cells by microscopic analysis. Part I should be performed by the instructor or by the class as a group during the laboratory session. Eight students or eight teams of students should then perform parts II and III.

I. Isolation of Nuclei

1. Place about one-half of the thymus (7-8 grams) into a 500ml beaker and cut the tissue into small (~1cm³) sections with scissors. Return the remaining thymus to the freezer.

relatedness of the proteins in sera samples can be compared both in terms of the number of cross-reactive proteins and in terms of their electrophoretic mobilities.

The first step in Western Blotting is to electrophoretically separate the serum protein samples in agarose gels. Next, the gel is placed against a specialized membrane made of nitrocellulose. The proteins are then forced out of the gel by applying gentle pressure and are trapped on the nitrocellulose membrane. As a result, a replica (blot) of the electrophoretically separated proteins is produced on the nitrocellulose membrane (see Figure 1-2).

Figure 1-2. Transfer Procedure for the Western Press-Blot.

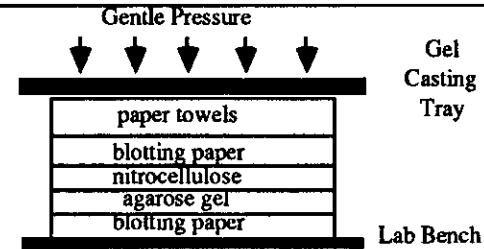
1. Run agarose gel.

2. Build "sandwich" consisting of gel casting tray, paper towel, blotting paper, gel, and nitrocellulose membrane.

3. Transfer separated proteins to nitrocellulose membrane by applying gentle pressure on the gel casting tray.

4. Detect all proteins on the membrane with protein blot stain.

5. Detect specific proteins on the nitrocellulose membrane with immunological techniques.



The next step is to incubate the membrane with antibodies which react with the proteins trapped on the membrane. In the present experiment, you will use antibodies that were generated in rabbits against cow serum albumin and cow gamma globulins. These antibodies will bind to the membrane-trapped cow albumin and cow gamma globulins and to the proteins from the other species that are structurally related to the cow proteins. Gelatin is added to the membrane before and during antibody incubation in order to minimize non-specific protein-antibody interaction.

Since the antigen-antibody complexes are not colored, they must be treated in some way in order to visualize them. A commonly used method involves the coupling of a color producing enzyme to the antibody. Enzymes which catalyze the

An understanding of the eukaryotic cell at the molecular level requires isolating intact organelles. To accomplish this goal, techniques are required for dis-isolating intact organelles. Cells can be broken open in various ways: by rupturing cells in a controlled fashion. Cells can be broken open in various ways: by mechanically shocking them. If carefully performed, disruption procedures reduce the plasma membrane, by osmotic shock or by addition of detergents that dissolve the extracellular matrix (called a homogenate) containing soluble components, plasma membrane fragments and intact organelles.

Nuclei are the largest organelles in a homogenate and can be separated from the other components by relatively low speed centrifugation. Following centrifugation, nuclei can be recovered as a pellet on the bottom of the centrifugation tube. These nuclei should retain the characteristic structure of those found in intact cells. The basic steps for the preparation of nuclei are shown in Figure 2-2.

B. CELL FRACTIONATION

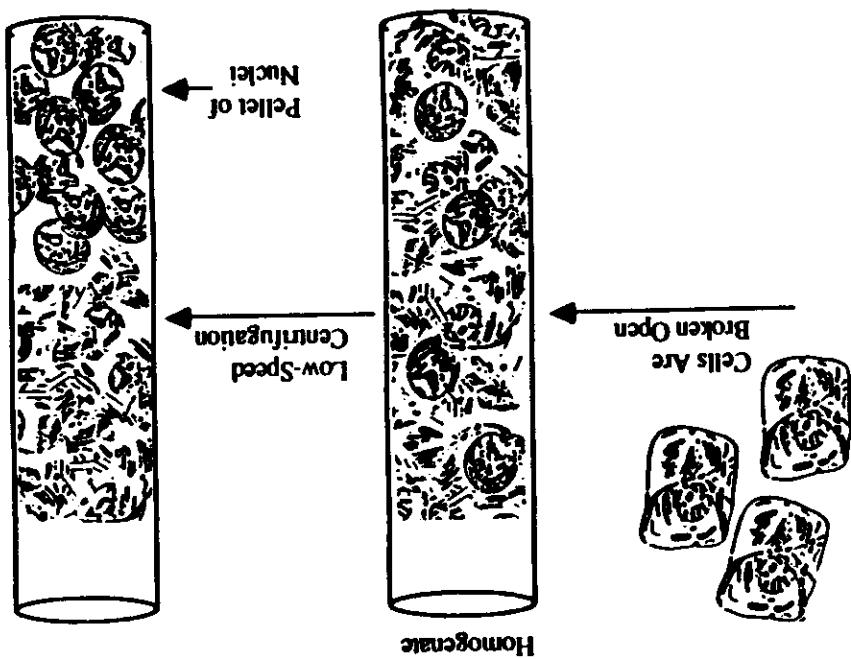


Figure 2-2. Isolation of Nuclei.

Nuclei are the largest organelles in a homogenate and can be separated from the other components by relatively low speed centrifugation. Following centrifugation, nuclei can be recovered as a pellet on the bottom of the centrifugation tube. These nuclei should retain the characteristic structure of those found in intact cells. The basic steps for the preparation of nuclei are shown in Figure 2-2.

An understanding of the eukaryotic cell at the molecular level requires isolating intact organelles. To accomplish this goal, techniques are required for disrupting cells in a controlled fashion. Cells can be broken open in various ways: by mechanical action, by osmotic shock or by addition of detergents that dissolve plasma membrane. If carefully performed, disruption procedures reduce cells to an extracellular homogeneous mixture containing soluble components, plasma membrane fragments and intact organelles.

In this laboratory, you will identify specific problems in serum and then determine the evolutionary relationships of these proteins by using the western blot procedure. A brief description of these proteins is given on the following page.

Serum contains a variety of small molecular weight components as well as hundreds of different serum proteins. Serum proteins, such as the antibodies, are important in fighting disease. Other proteins in serum, such as albumin and transferrin, function as carrier molecules for the transport of small molecular weight compounds such as metals, fatty acids, amino acids, hormones and drugs.

Blood is a remarkable tissue containing cellular elements (erythrocytes, leukocytes and platelets) suspended in a liquid medium called plasma. Whole blood lacks certain proteins (fibronogen and some clotting factors) that are involved in the clotting process.

III. SERUM PROTEINS

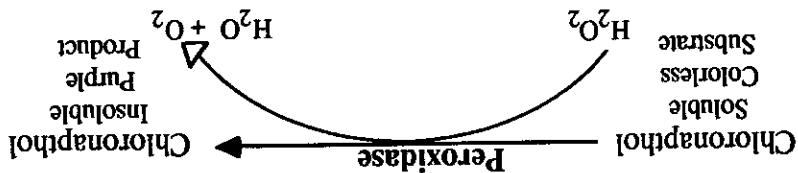
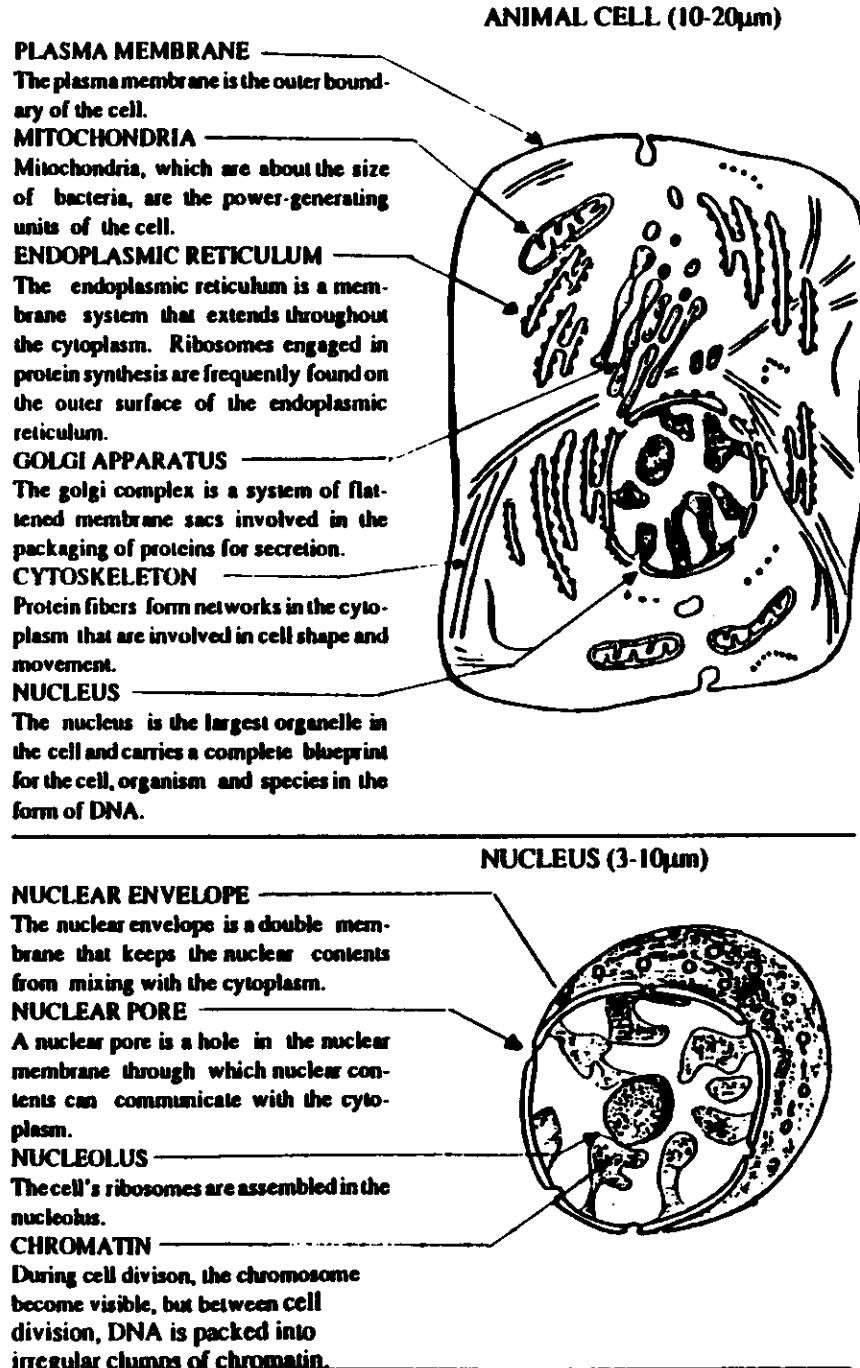


Figure 1-3. The Reaction Catalyzed by Horseradish Peroxidase.

reaction of soluble, colorless substances to insoluble, colored products are often coupled to antibodies to permit visualization. In this experiment, the antibodies you will use have been coupled to Horseradish Peroxidase (HRP) which catalyzes the reaction diagrammed in Figure 1-3. Following incubation of the membrane with the antibody, the final step in the Western Blotting procedure is to incubate the blot in a color development solution containing hydrogen peroxide and 4-chloro-1-naphthol. The immobilized HRP then converts the 4-chloro-1-naphthol to an insoluble purple product which is deposited at the site of the antigen bands and allows antigen visualization.

Figure 2-1. A Generalized Animal Cell and Nucleus.



Serum Albumin - Serum Albumin is the major protein found in serum and this protein transports a number of small molecules in blood. Although albumin is not naturally colored, the protein binds to bromophenol blue. This feature will enable you to identify albumin after electrophoresis since bromophenol blue has been added to the samples and some of this dye will remain bound to the albumin during the electrophoretic run.

Transferrin - Transferrin binds iron and this protein transports iron in the circulation.

Gamma Globulins - The numerous antibodies in blood are found in the gamma globulin fraction of serum.

Objective: To identify albumin, transferrin, and gamma globulins in serum and to study the evolutionary relationships of albumin and gamma globulins in vertebrates.

Laboratory Schedule: This experiment was designed such that two students will work together, sharing one gel and one blot. If students work in pairs, the samples of four students should be analyzed on one gel and one blot. The entire procedure can be performed in one 3-hour laboratory period. Alternatively, steps I - IV can be done in one two-hour lab and steps V and VI can be performed during the next lab session.

Materials Provided

Goat Serum
Sheep Serum
Cow Serum
Horse Serum
Chicken Serum
Cow Transferrin
Cow Gamma Globulins
Cow Albumin

Antibodies to Cow Albumin-HRP - This antibody was prepared by injecting rabbits with cow serum albumin. It is coupled to the enzyme HRP (Horse Radish Peroxidase).

Antibodies to Cow Gamma Globulins-HRP - This antibody was prepared by injecting rabbits with cow gamma globulins. It is coupled to the enzyme HRP.

Nitrocellulose (4 sheets)

Blotting paper (16 sheets)

*Gelatin solution

*TBS (Tris-Buffer Saline)

*TBS + NP40 (Tris-Buffer Saline + Nonidet (P40))

*Color Development Solution (contains chloronaphthol and hydrogen peroxide dissolved in development buffer - this solution should be prepared immediately before use.)

Cells are frequently classified into two basic types: prokaryotic and eukaryotic. The prokaryotic cell, so named because it lacks a discrete nucleus, is found in bacteria and blue-green algae. Prokaryotic cells have relatively simple internal structures, although they maintain their metabolic activities are as complex as those in humans. The eukaryotic plan is observed in the algae, fungi, protists, and the cells of higher animals and plants. The eukaryotic cell, by definition, has a nucleus that contains most of the cell's DNA. The nucleus is enclosed by a double membrane, the nuclear envelope, that separates nuclear components from the cytoplasm. The nucleus also contains a number of other specialized internal structures not found in prokaryotes including mitochondria, chloroplasts, and a rich array of internal fibers and membranes. A diagram of a typical animal cell is given in Figure 2-1.

The genetic material in a prokaryotic cell usually consists of a single circular molecule of DNA that is compacted within the cell interior. In contrast, DNA in the eukaryotic nucleus is thought to consist of a number of chromosomes, and the DNA molecule of each chromosome is partitioned into a single linear molecule. Human cells contain 46 chromosomes and the average extended length of each chromosomal DNA molecule is about 4 centimeters. Therefore, the length of DNA molecules in one human cell is nearly 2 meters (46 chromosomes \times 4 cm = 1.8 meters). This length is about 200 times greater than the distance from the earth to the sun! Clearly the DNA must be condensed or folded in some manner to fit within the confines of the nucleus.

In the nucleus of a typical eukaryotic cell, individual chromosomes can be identified only during cell division. However, a eukaryotic cell spends most of its life cycle in interphase, which is the stage between cell divisions. In interphase, the histones, which are basic in nature because they have large amounts of the basic amino acids arginine and lysine. These basic proteins associate with the acidic DNA backbone, folding the long DNA molecules into chromatin and compacted chromatosomes. When these proteins are dissociated (removed) from chromatin, the DNA molecules unfold, resulting in an increase in the viscosity of the solution.

A. THE EUKARYOTIC CELL

Background Information

Experiment (B1-2). Cell Fractionation and DNA Isolation

Materials not provided

Gloves A water bath maintained at 37°C 4 paper towels 4 razor blades 4 scissors

4 containers for blot washing - These containers should hold about 100 ml and be used for all steps described below except the antibody reactions.

*Prepared as described in the Instructor Guide.

The sera samples, albumin, transferrin, and gamma globulins are dissolved in electrophoresis sample buffer which contains glycerol and bromophenol blue.

*Ponceto S - Protein "Blot" Stain 4 plastic petri dishes for blot incubation - These dishes should be used for the antibody reaction described under Part V.

- 1 Cow Gamma Globulins Sample Well
- 2 Cow Serum Albumin Sample
- 3 Cow Transferrin Group I
- 4 Cow Serum Group 2
- 5 Goat Serum Sheep Serum Horse Serum Chicken Serum
- 6
- 7
- 8

- 1 Load 15 µl of the following samples into the sample wells.
- 2 Boil the agarose and then pour the gel as described on page 14.
- 3 Prepare one 0.8% agarose gel, dispense 15 ml of electrophoresis buffer (Tris-Glycine, pH 8.6) into a 25 ml glass test tube and add 0.12 grams of agarose. Four gels will be used in the experiment.
- 4 Electrophoresis

1	Cow Gamma Globulins	Sample
2	Cow Serum Albumin	
3	Cow Transferrin	
4	Cow Serum	
5	Goat Serum	Horse Serum
6	Sheep Serum	Chicken Serum
7		
8		

4. A single additional band containing the segment of phage lambda DNA should be present in lanes 3 and 7 of your gel. Identify this band. This band should be the same length as one of the bands in the EcoR1 digest of phage lambda DNA on lanes 1 and 5. Record the length of the band containing the segment of lambda DNA (lanes 3 and 7) in the Table below.

5. Two bands, in addition to the pUC18 band, should be present on lanes 4 and 8 of your gel. These bands should be the same length as two of the bands in the EcoR1 and Bam H1 digest of phage lambda DNA on lanes 2 and 6. Identify these bands and record their lengths in the Table below.

6. With the aid of Figure 2, determine the precise region in the Lambda genome that is contained within the recombinant plasmid. Record the coordinates for this region (in base-pairs from the left end of the genome) in the Table below.

Length of the EcoR1 segment of Lambda DNA in the plasmid-lambda (lanes 3 and 7)	Length of the Lambda DNA segments in the EcoR1 and Bam H1 digest	Coordinates of the region in the lambda genome that is contained within plasmid-lambda
---	--	--

7. With the aid of Figure 2, describe one function of the lambda DNA that is contained within plasmid-lambda.

8. In DNA of uniform composition, a specific hexanucleotide occurs by chance once in every 4016 base-pairs. The recognition sequence for EcoR1 is a hexanucleotide (see Part A-1, Table 1). How many restriction sites for EcoR1 would you expect to exist in lambda DNA if the phage DNA were of uniform composition? There are few restriction sites for a number of enzymes including EcoR1 and HpaI in the left 20 kilobases of lambda DNA. Explain. (Hint: The left region of lambda DNA is rich in G+C base pairs.)

2. During the electrophoretic run, perform Step III-1 (below).
3. Electrophorese until the albumin (blue bands) in the sera samples and in lane 2 has migrated to within 1.5cm of the positive end of the gel. At 170 volts, this should take about 50 minutes.

III. Preparation of the "Western Press - Blot"

1. During the electrophoretic run, perform the following tasks:

- A. Wet one sheet of nitrocellulose by floating it in a container with about 20ml of distilled water. After wetting, pour off the water and replace with 5-10ml of TBS. In a separate container, wet two sheets of blotting paper with 5ml of TBS.

Note: Gloves should be worn when handling nitrocellulose to prevent transfer of proteins from your hands to the membrane. If gloves are not available, use forceps. Touch only the edges of the membranes with gloves or forceps.

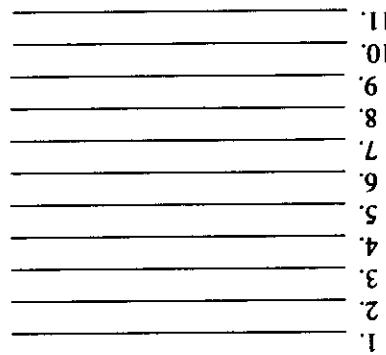
- B. Cut 6 paper towels (to about 5cm x 7cm) to form a stack that is 1-2cm high.
- C. Clean a small section of your laboratory bench top.
2. After electrophoresis, build a "sandwich" on your clean bench top by sequentially overlying each of the following materials. Smooth with your gloved index finger after each addition except after step C to eliminate air bubbles between layers.
 - A. One sheet of wet blotting paper
 - B. Agarose gel (place the gel upside down onto the blotting paper so that the nitrocellulose will be in contact with the bottom of the gel).
 - C. One nitrocellulose membrane - After the addition of the nitrocellulose membrane, trim a small corner of the membrane at the bottom of lane 1. This marking will enable you to identify the positions of the gel lanes on your blot.
 - D. One sheet of wet blotting paper.
 - E. One sheet of dry blotting paper.
 - F. One sheet of dry blotting paper.
 - G. One stack (1-2cm) of dry paper towels.

4. After completion of your diagram, place the blot in 25ml of gelatin solution and incubate at 37°C for 10 minutes. Replace the solution with 25ml of fresh gelatin and incubate for an additional 5 minutes. The gelatin serves to block sites on the nitrocellulose that are not occupied by protein. The dyes will dissociate from the proteins during this step and the blots should appear colorless at the end of the incubation.

Data Analysis and Study Questions

1. Place your gel over a light source. The sizes of the DNA bands in the EcoRI digestes elutedophoresed on lanes 1 and 5 should be 21.2, 7.4, 5.8, 5.6, 4.8 and 3.5 kilobase pairs (see Figure 2). Identify these bands on your gel. From the map of phage lambda given in Figure 2, identify the DNA bands that contain the genes for the head proteins and the genes that control lysis of the host cell.
2. Maps of the restriction sites for EcoRI and Bam HI in lambda DNA are given in Figure 2. With this information:

 - A. Calculate the length of DNA fragments that should have been produced when lambda DNA was digested with both EcoRI and BamHI. List these values below and indicate the two fragments that contain the cohesive ends.
 - B. Identify the above fragments on lanes 2 and 6 of your gel.



DNA Fragment Length
(Kilobase-pairs)

Kilobase-pairs

A. Preparing the DNA samples

1. Number four small (0.5ml) tubes 1 to 4 with a water-proof marking pen.
2. Place 10 µl of the EcoR1-buffer solution into tubes 1 and 3, and 10 µl of the EcoR1 + Bam H1 mixture into tubes 2 and 4.
3. Add 5 µl of lambda phage DNA to tubes 1 and 2 and 5µl of plasmid lambda to tubes 3 and 4. Gently tap the tubes with the tip of your index finger to mix the solutions. Incubate the tubes for 60 minutes at 37°C.
4. During this incubation, prepare 1.2% agarose gels as described in Part A of this manual.
5. At the end of the 60 minute incubation period, add 5µl of electrophoresis sample buffer to each of the four tubes.
6. Transfer the tube to the 65°C water bath and, after 5 minutes, place the tubes in an ice bath. This heat treatment will inactivate the restriction enzymes and will separate the two fragments containing the cohesive ends of phage lambda.

B. Electrophoresis

1. Load 15µl of the following samples from the above section into the sample wells.

Sample Well	Sample
1	Tube 1
2	Tube 2
3	Tube 3
4	Tube 4
5	Tube 1
6	Tube 2
7	Tube 3
8	Tube 4

2. Electrophorese until the bromophenol blue in the samples has migrated to within 1 mm of the positive electrode end of the gel.
3. Remove the gels from the unit and stain them as described in Part A of this manual.

5. The blots can be processed immediately as described below or they can be stored in gelatin for a few days in the refrigerator. If stored in the refrigerator, allow them to warm to 37°C before continuing.

V. Antibody Reaction

Two of the blots will be incubated with antibodies to cow albumin while the remaining two blots will be incubated with the antibodies to gamma globulins. The four blots will be compared at the end of the experiment.

1. Place 50µl of either the cow albumin antibody **or** 50µl of the cow gamma globulin antibody into a petri dish. Add 6ml of gelatin to each dish. Transfer the blot to the dish and gently swirl the dish to ensure that all surfaces of the blot are exposed to the antibody solution.
 2. Place the lid on the dish, and float the dish in a water bath at 37°C for 25 minutes.
- Note: Great care should be taken not to bump the dishes during the incubation.
3. Transfer the blot to a suitable small container (e.g. a clean gel staining tray) and wash for 2 minutes each in 40 ml of the following solutions. Manual rocking or shaking of the container should be performed during these washes.

1. TBS+NP40
2. TBS+NP40
3. TBS+NP40
4. TBS
5. TBS

4. While the blots are washing, the instructor should prepare the Color Development Solution by adding 7ml of Color Development Buffer, 0.5ml of hydrogen peroxide and 5ml chloronaphthol to 130ml of water.

VI. Color Development Reaction

1. Place 30ml of the Color Development Solution into a small container and transfer the blot to the solution. Gently rock blot in color development solution until purple bands appear. This should take about 5-15 minutes. Rinse the blot in water and carefully examine both sides for purple bands. Blots may be stored protected from heat and light (between 2 sheets of black construction paper, for example).

Data Analysis and Study Questions

- Objective**
- Phage lambda DNA and a recombinant plasmid containing a segment of the phage lambda genome are provided as the starting point for this exercise. You will digest these DNAs with EcoRI and Bam HI and then analyze the fragments by electrophoresis. Determine the size of the fragments that is contained within the plasmid.
- Materials**
1. Phage Lambda DNA
- The samples below are provided for the analysis.
2. Plasmid-Lambda: The recombinant plasmid was prepared by inserting an EcoRI segment of phage lambda DNA into the EcoRI site of plasmid pUC18 as outlined in Figure 1. Plasmid pUC18 without the insert is 2.7 kilobase-pairs in length.
3. EcoRI: The restriction enzyme to be used in this laboratory should be made up immediately before the laboratory session as described in the Instructor Guide.
4. ECO RI + Bam HI: This mixture contains both restriction enzymes suspended in a nucleic acid digestion buffer and should be made up as described in the Instructor Guide.
5. Electrophoresis sample buffer: The buffer contains electrophoresis buffer, glycerol, and bromophenol blue.
- Materials Not Provided**
1. Water baths for tube incubation maintained at 37°C and 65°C: A beaker containing water heated to 65°C with a Bunsen burner can serve as the latter bath.
2. Ice bath: Ice chips in a beaker can be used.
3. Microtubes (0.5ml) and tube racks.
4. Agarose, electrophoresis buffer, and gel stain (See Instructor Guide)
- Procedure**
- The experiment was designed for 8 students working individually or 16 students working in teams of two.

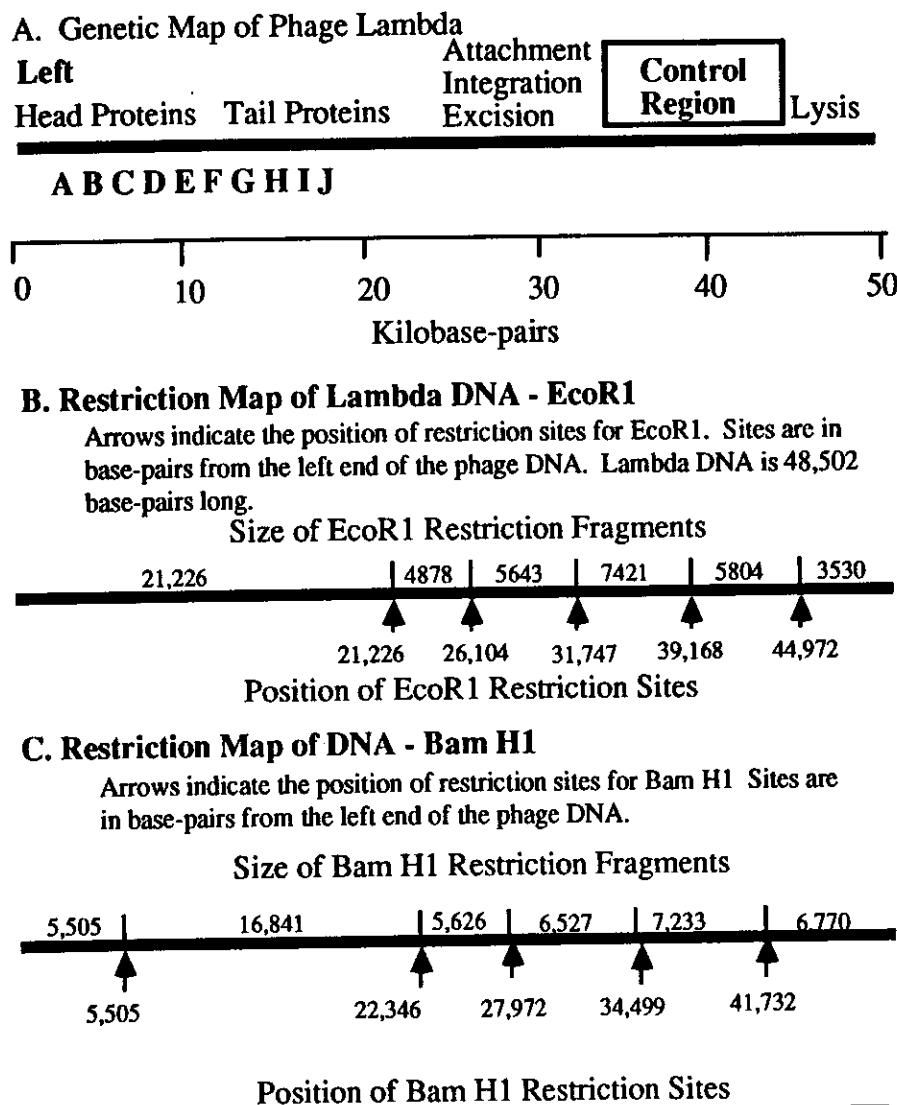
LANES	1	2	3	4	5	6	7	8
ALBUMIN								
ANTIBODY TO GAMMA GLOBULINS								

1. Examine your blot carefully and compare your results to the results of your classmates. In the space provided below, prepare diagrams of the indicated bands.

2. Compare the reaction of the antibodies to cow albumin and gamma globulins from the various vertebrates. Which species show the greatest similarity to cow with respect to the reaction? Which shows the least?

3. How does this analysis compare with the traditional taxonomic relationships reported for these animals? You may need to consult a zoology or comparative anatomy text book to determine what is known about the relationships between cow, sheep, goat, and horse.
4. Describe how your results would have been affected if the antibody had been made in rabbits against duck albumin.

Figure 1. Genetic and Restriction Map of Phage Lambda DNA.



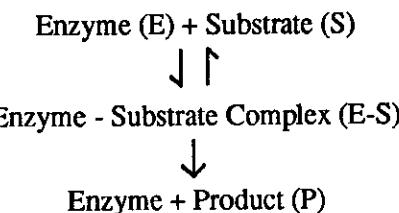
IND-5. Specificity of Albumin Binding

Background Information

A. INTERACTION BETWEEN MOLECULES IN BIOLOGICAL SYSTEMS

One of the most important principles of modern biology is that two molecules with complementary surfaces tend to bind or stick together, whereas molecules without such surfaces do not. This principle can be illustrated by considering the specificity of enzyme action. Enzymes accelerate the velocity of virtually all reactions that occur in biological systems, including those involved in breakdown, synthesis and chemical transfers. In so doing, they are responsible for performing essentially all the changes associated with life processes.

The general expression frequently used to describe an enzyme reaction is:



Certain important features of the nature of enzyme reactions are evident from this diagram:

1. The term substrate refers to the compound that is acted upon by the enzyme. In general, enzymes exhibit a high degree of substrate specificity in that they usually catalyze only a single chemical reaction.
2. The enzyme binds to the substrate to form an enzyme-substrate complex. This interaction is responsible for the specificity of enzyme action, since only those compounds that "fit" into the substrate binding site can be acted upon by the enzyme.
3. The enzyme is not destroyed during the reaction but rather is set free after the formation of the end product. Thus, the liberated enzyme is available to combine with more substrate to produce more product.

The binding of an enzyme to its substrate is only one example of the many specific molecular interactions that occur in biological systems. Analogous binding processes occur when an antibody binds antigen or when a hormone binds to its receptor. In each case, the molecule that binds to the protein molecule is called a ligand and the region of the protein that associates with the ligand is known as the binding site. Table 1 shows a list of some of the major ligand-protein interactions that occur in biological systems.

II. Phage Lambda

Viruses are a unique group of parasitic organisms that grow only in the cells of bacteria, plants and animals. A virus that infects bacteria is called a bacteriophage or simply a phage. Viruses have provided invaluable tools for the study of molecular biology because they possess the essential properties of life yet are simpler than bacteria or eukaryotic cells in their structures and life cycles.

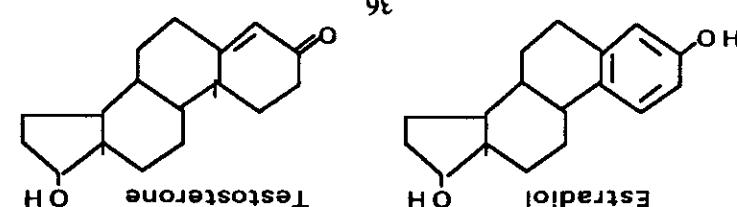
The DNA molecule contains 48,502 base-pairs (molecular weight 3×10^7) head. A single molecule of double-stranded DNA is located in the core of the phage. The DNA molecule contains 50 different phage proteins. The sequence of nucleotides along the entire lambda genome is known and the nucleotide sequences that code for approximately 50 different phage proteins. The sequence of nucleotides of the major control regions for transcription and replication have been determined. Because of the vast amount of information about the biology of this denatured. When lambda has become a common cloning vector in genetic engineering.

When lambda has been injected into the *E. coli* host, the phage DNA is injected into the bacterial cell. The bacterial cell then releases newly synthesized phage particles. About one hundred lambda particles are released into the medium when the formed DNA molecules are packaged into newly synthesized phage particles. The bacterial cell is broken open (lysed). As a result of this lytic infection, the bacterial DNA is replicated as part of the bacterial chromosome. However, when the lambda DNA is replicated from the host chromosome and becomes a normal coil DNA. After integration, the bacterial cell behaves normally and the lambda genome is excised from the host chromosome and becomes a normal coil DNA. A map of phage lambda DNA shows a map of phage lambda DNA. The genes of the phage are clustered according to their function and the DNA may be divided into three regions. The left-hand region includes all the genes (A through J) whose products are necessary to produce phage head and tail proteins and to package the DNA into the virions. The central region contains elements involved in integration of the DNA into the *E. coli* chromosome. The remaining portion of the genome includes the genes necessary for transcription and replication, and the genes necessary for cell lysis.

B. CHARACTERISTICS OF LIGAND-PROTEIN INTERACTIONS

Protein	Ligand
Enzyme	Substrate
Antibody	Antigen
Receptor	Hormone
Glycoprotein	Leucin
Serum Albumin	General including
Specific dyes	Certain synthetic dyes

Table 1. Ligand-Protein Interactions



Ligand Structure and Binding Specificity - The binding of a ligand to a protein is a specific process which is highly dependent on the structure of the ligand. For example, the two major groups of sex steroid hormones are the estrogens and the androgens which are found in males and females, respectively. The structures of the major estrogen (called estradiol) and androgen (testosterone) are shown on the right. Although the structures of these two hormones are quite similar, their actions are very different. Estradiol promotes feminizing effects such as breast growth in both males and females while testosterone causes masculine reactions in males. Estrogens which are found in females, especially those with breast cancer, are responsible for many of the symptoms associated with breast cancer. For example, the breast tissue is very sensitive to estrogen and the biological response produced by these hormones is due to estrogen and androgen receptor proteins produced by tissues that respond to these sex steroids. The estrogen receptor protein is found in tissues that respond to estrogen and this protein specifically to testosterone. By contrast, androgen receptor proteins are found in steroid but not in testosterones. Consistently, the differences in male and female phenotypes.

In the ligand binding specificities of these receptor proteins, the differences in the ligand like testosterone but not estrogens. Consistently, the differences androgen receptor proteins are found in androgen target tissues and these receptors bind androgens like testosterone but not to estrogens. By contrast, the differences in the ligand binding specificities of these receptor proteins are found in tissues that respond to estrogen and androgen receptor proteins produced by these hormones is due to estrogen and the biological responses produced by these hormones in both sexes. The differences in including muscular development and hair growth in both sexes. The differences in growth in both males and females while testosterone causes masculine reactions below. Although the structures of these two hormones are quite similar, their actions are very different. Estradiol promotes feminizing effects such as breast growth in both males and females while testosterone causes masculine reactions in males. Estrogen which is highly dependent on the structure of the ligand is shown on the right. Although the structures of these two hormones are the estrogens and the androgens which are found in males and females, especially those with breast cancer, are responsible for many of the symptoms associated with breast cancer. For example, the breast tissue is very sensitive to estrogen and the biological response produced by these hormones is due to estrogen and androgen receptor proteins produced by tissues that respond to these sex steroids. The estrogen receptor protein is found in tissues that respond to testosterone. By contrast, androgen receptor proteins are found in steroid but not in testosterones. Consistently, the differences in male and female phenotypes.

the plasmid using the restriction enzymes EcoR1 and Bam H1. The segment of foreign DNA was derived from a virus called phage lambda which is described below.

Figure 1. Preparation of a Recombinant Plasmid

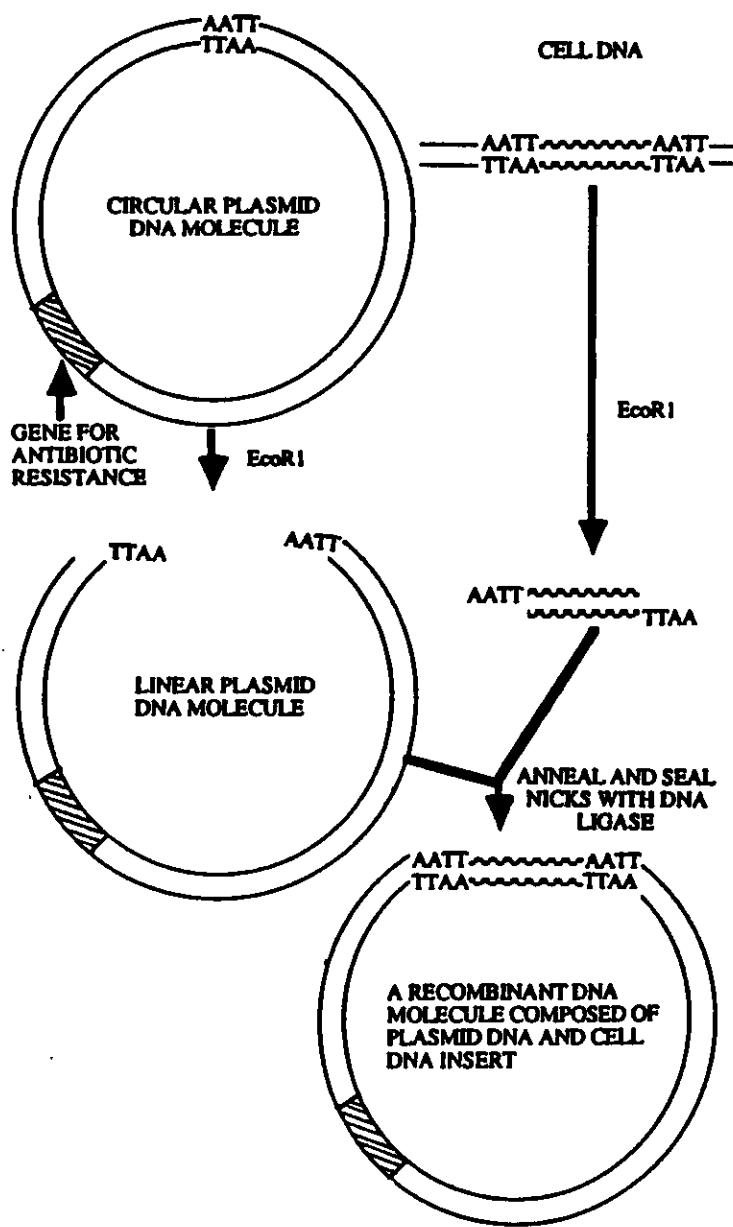


Figure 1. Saturation of Protein by Ligand

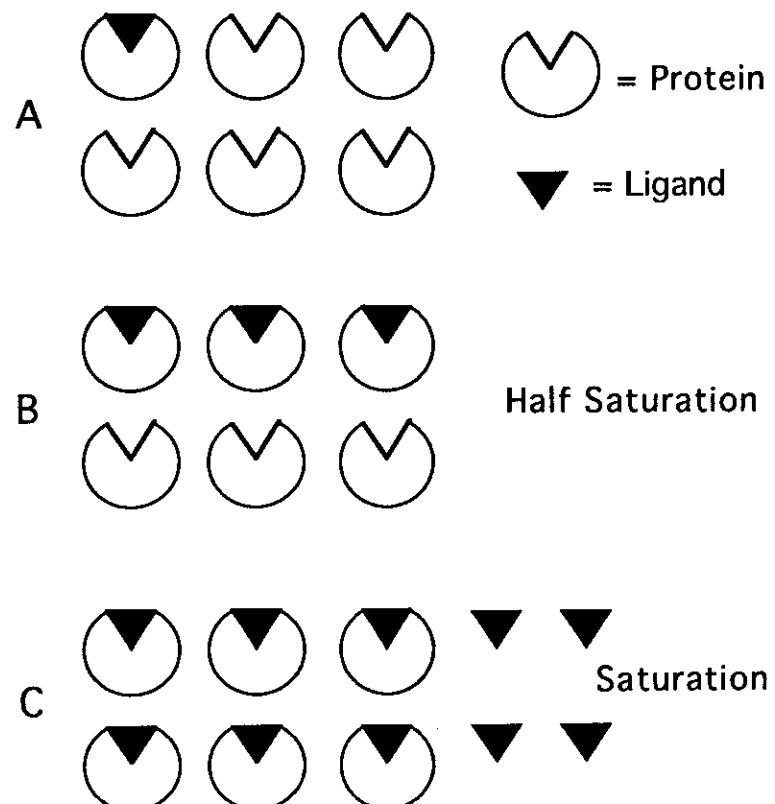
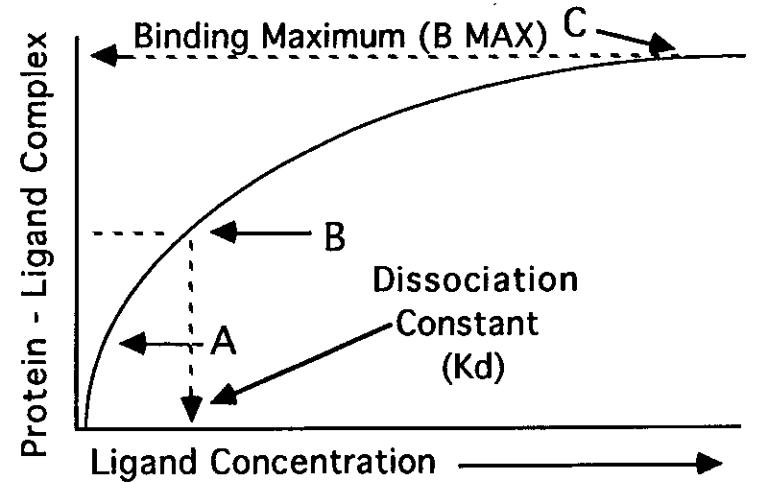


Table 1. Properties of Three Restriction Enzymes

Restriction Nucleases	Bacterial Source	Recognilicin Sequences and Mode of Cleavage	Hpa I
		Cuts on lines of symmetry	-G-T-T-A-A-C- -G-T-T-A-A-C- -C-A-A-T-T-G- -C-A-A-T-T-G-
		Blunt ends	
EcoRI	<i>Escherichia coli</i>	Staggered Cleavage	-C-T-T-A-A -G-A-A-T-T-C -C-T-T-A-A-G- -C-T-T-A-A-G- -G-T-T-A-T-C- -G-T-T-A-T-C- -C-A-A-T-T-C- -C-A-A-T-T-G-
		Cohesive (Sticky) Ends	
Bam HI	<i>Bacillus amyloliquefaciens</i>	Staggered Cleavage	-C-T-A-G -G-A-T-C-C -C-C-T-A-G -C-C-T-A-G- -G-A-T-C-C -C-C-T-A-G- -C-C-T-A-G-
		Cohesive (Sticky) Ends	

In this laboratory, you will be given a recombinant plasmid which contains a segment of foreign DNA inserted into the EcoRI site. The plasmid was prepared essentially as described in Figure 1. You will then characterize the foreign DNA segment of foreign DNA.

dividing bacteria to produce an enormous number of copies of the original DNA fragment. At the end of the propagation period, the hybrid plasmid molecules are purified from the bacteria. Copies of the original DNA fragments can then be recovered by cleavage of the recombinant plasmid with EcoRI.

Cohesive (Sticky) Ends

-C-C-T-A-G

Staggered Cleavage

-C-C-T-A-G-

Cohesive (Sticky) Ends

Staggered Cleavage

-C-T-T-A-G-

.....

Cuts on lines of symmetry

C-A-A-T-G-

Recognition Sequent

Table 1. Properties of Three Restriction Enzymes

In today's laboratory, you will use an electrophoretic procedure to study the binding interactions that occur between serum albumin and three synthetic dyes. The dyes are called Bromophenol Blue, Ponceau S, and Orange G. The basis for this procedure is the observation that the free dyes not bound to serum albumin move faster than albumin or dyes bound to albumin as shown below. This separation will enable you to distinguish between albumin bound dye and free not associated with the protein. The exercise was designed for eight groups of students and each group will perform a different experiment. At the end of the electrophoretic run, each group will present a five minute oral presentation on the results of their experiment.

Whole blood, or plasma, clots upon standing and if the clot is removed, the remaining straw-colored fluid is called serum. Serum contains a variety of small molecular weight components as well as hundreds of different serum proteins. The major protein in serum is albumin, which functions as a carrier molecule for the transport of certain small molecular weight compounds in blood. Molecules that bind to serum albumin include bilirubin, fatty acids, hormones, and some synthetic

C. DESCRIPTION OF THIS LABORATORY EXERCISE

Saturation of Protein by Ligand - The biological response to a steroid hormone is a saturable phenomena. That is, high doses of a steroid hormone produce a maximum response which is not enhanced by additional hormone. Likewise, the biological effects produced by essentially all ligand-protein interactions wise, the amount of protein bound to ligand as a function of ligand concentration. The curve obtained for nearly all protein-ligand pairs is a hyperbola like the one shown in the figure. As noted in the figure, an increase of ligand like the one shown in the figure. This effect is illustrated in Figure 1 which can be saturated with excess ligand. This effect is illustrated in Figure 1 which shows a graph of the amount of protein bound to ligand versus ligand concentration. The curve increases rapidly at first and then levels off. At a low ligand concentration, the curve increases rapidly. As the concentration of ligand continues to rise, the increase in binding slows and the curve flattens. At a maximum binding, the protein is saturated with ligand and additional binding does not occur upon a further increase in ligand concentration. The concentration of ligand required to yield half maximal binding defines the dissociation constant (K_d) which provides an index of the affinity of the protein for the ligand. Thus, a K_d of 0.1 M/L of ligand would indicate that the protein binding site is half saturated with ligand when the ligand concentration is present at that concentration. Such a protein has a very low affinity for its ligand. In contrast, a K_d of 10^{-8} M/L indicates that the protein has a high affinity since it is half saturated at this low ligand concentration. Most specific protein-ligand interactions in biological systems are characterized by K_d values ranging from 10^{-8} to 10^{-12} M/L.

Experiment 1002. Analysis of a Genome Segment

Background Information

I. Preparation of a Recombinant Plasmid

Plasmids are small circular DNA molecules that exist apart from the chromosomes in most bacterial species. Plasmids, as well as certain viruses, are extraordinarily useful tools for the molecular biologist, because they serve as gene-carrier molecules called cloning vectors. A basic procedure of recombinant DNA technology consists of joining a gene of interest to vector DNA to form a hybrid or recombinant molecule that is able to replicate in bacteria. In order to prepare a recombinant DNA molecule, a procedure is required for cutting cloning vectors and cellular DNA molecules at precise positions.

Restriction nucleases are powerful tools used in recombinant DNA technology because they cut DNA at specific sites. These enzymes are produced chiefly by bacterial species in which they serve to degrade invading foreign DNA within the bacterial cell. Most restriction enzymes recognize a specific sequence of four to six nucleotides in DNA and each will cut a long DNA double helix into a series of discrete pieces known as restriction fragments.

Typically, the restriction sites for a given enzyme are hundreds to thousands of base-pairs apart so that the fragments generated are hundreds to thousands of base-pairs long. More than 200 different restriction nucleases are now commercially available. General properties of three of these enzymes are given in Table 1. It should be noted that some restriction nucleases (e.g., EcoR1 and Bam H1) produce a staggered cleavage that creates sticky, or cohesive, single-stranded ends on the cut molecules. These cohesive ends are very important in recombinant DNA procedures because they enable any two DNA fragments to be linked together by complementary base pairing at their ends, provided that they were generated with the same restriction enzyme.

Figure 1 illustrates one basic procedure by which cellular DNA from essentially any source can be amplified by recombinant DNA techniques. First, a plasmid is cleaved at a single site by a restriction nuclease, such as EcoR1, that produces cohesive ends on the plasmid DNA. The cellular DNA to be cloned is cleaved with the same enzyme, and fragments of the cellular DNA are annealed to the plasmid DNA by complementary base-pairing at the cohesive ends of the DNA molecules. The newly formed joints are sealed with an enzyme called DNA ligase, which forms covalent bonds between the ends of each DNA molecule. The recombinant DNA molecules are then introduced into *E. coli* by transformation, and the bacteria are grown in the presence of an antibiotic. The hybrid plasmid can replicate in the

Materials Provided

Dilution Buffer - The buffer contains 0.05M NaCl, 20% Glycerol, and 10mM Tris pH 8.0

Bovine Serum Albumin

Rabbit Hemoglobin

Sodium Dodecyl Sulfate (SDS) - 10%

Bromophenol Blue

Orange G

Ponceau S

Proteins and dyes are suspended in dilution buffer and the concentration of each dye is 0.01M.

Procedure

Each group should select to perform one of the following experiments.

1. Prepare 1.2% agarose gels as described in the first part of this manual.
2. While the gels are cooling, each group should number four small (0.5ml) tubes 1-4 with a water proof marking pen.
3. Add dilution buffer, bovine serum albumin(BSA), and dyes to the four tube as indicated below.

Group -1. Albumin Binding is Saturable and Ligand Specific

Tube #	Buffer	BSA	Bromophenol Blue
1	20µl	0	10µl
2	15µl	10µl	5µl
3	10µl	10µl	10µl
4	0	10µl	20µl

Group - 2. Albumin Binding is Saturable and Ligand Specific

Tube #	Buffer	BSA	Ponceau S
1	20µl	0	10µl
2	15µl	10µl	5µl
3	10µl	10µl	10µl
4	0	10µl	20µl

Group - 3. Albumin Binding is Saturable and Ligand Specific

Tube #	Buffer	BSA	Orange G
1	20µl	0	10µl
2	15µl	10µl	5µl
3	10µl	10µl	10µl
4	0	10µl	20µl

Group - 4. Ligand Binding is Protein Specific

Tube #	Buffer	Protein	Bromophenol Blue	Study Questions and Analysis
1	20μl	0	5μl	1. Describe the basic properties of DNA that are responsible for fiber formation when alcohol is added to a solution that contains native (double-stranded) DNA.
2	10μl	10μl Hemoglobin	5μl	2. Describe the type of precipitate that is formed when alcohol is added to denatured (single stranded) DNA. Offer an explanation as to why this precipitate is different from that which is observed with native DNA.
3	20μl	0	5μl	3. Describe the action of DNase I on DNA and relate this effect to the results of your experiment in Section III.
4	10μl	10μl BSA	5μl	

Group - 5. Ligand Binding is Protein Specific

Tube #	Buffer	Protein	Poncaeu S	Group - 6. Ligand Binding is Dependent on the Native Structure of Albumin
1	20μl	0	5μl	
2	10μl	10μl Hemoglobin	5μl	
3	20μl	0	5μl	
4	10μl	10μl BSA	5μl	

Group - 6. Ligand Binding is Dependent on the Native Structure of Albumin

Tube #	Buffer	BSA	Dye	SDS	Group - 7. Ligand Binding is Reversible and Competitive
1	5μl	5μl Bromophenol Blue	0		
2	10μl	10μl Bromophenol Blue	5μl		
3	10μl	5μl Poncaeu S	0		
4	0	10μl Poncaeu S	5μl		

Group - 8. Ligand Binding is Reversible and Competitive

Tube #	Buffer	BSA	Poncaeu S	Bromophenol Blue	Group - 8. Ligand Binding is Reversible and Competitive
1	20μl	10μl	10μl	0	
2	15μl	10μl	10μl	5μl	
3	10μl	10μl	10μl	10μl	
4	0	10μl	10μl	20μl	

4. After no more fibers can be formed, remove the rod and examine the DNA fibers wound around it.

5. Rinse the vial with water.

6. (OPTIONAL)

The precipitation of DNA by alcohol is reversible. This can be illustrated by placing the rod containing the DNA fibers into a glass test tube that contains 2 mls of 1% NaCl. Shake the rod until the DNA fibers are in the solution. It takes about 1 day for the precipitated DNA to dissolve, and at this time, it can be respoiled as described above.

II. Analysis of Denatured (Single-Stranded) DNA

1. One member from each group should place 3ml of DNA solution into a glass test tube.

2. Place the tube in a boiling water bath and boil the contents for about 3 minutes.

3. Carefully transfer the contents of the tube into a glass vial and then place the vial in a beaker containing ice chips.

4. After a few minutes, remove the cooled vial from the beaker, add cold alcohol to it, and attempt to spool the DNA onto the rod as described above (Section I, 3-4).

5. Stir the contents of the vial with the rod in order to mix the DNA with the alcohol. Note and record the type of precipitate that is formed.

6. Rinse the vial with water.

III. Effects of DNase I on the Structure of DNA

1. One member from each group should place 3 ml of the DNA solution into the glass vial.

2. Using a small transfer pipet, add 50 μ l (about 4 drops) of the DNase I solution to the vial and mix the contents of the vial carefully.

3. After 10minutes, add cold alcohol to the vial and attempt to spool the DNA onto the rod as described above (Section I, 3-4).

4. Stir the contents of the vial with the rod in order to mix the DNA with the alcohol. Note and record the appearance of the mixed solution.

4. Load 15 μ l of each of your samples (Tubes # 1-4) into four adjacent sample wells. Two groups should share one gel.

5. Electrophorese for 10 minutes at 170 volts, and then turn off and disconnect the power supply.

6. Remove the lid from the electrophoresis unit and note the relative gel position of the albumin bound and free dyes used in your experiment.

7. Resume electrophoresis until the dyes that are not bound to albumin have migrated to within 1cm of the positive electrode end of the gel. During this time, prepare an outline of your presentation that will be given at the end of the electrophoretic run. The format below may help in the preparation of the outline.

8. At the end of the electrophoretic run, remove the gels from the unit. At this time, you should measure the distance (in ml) migrated by the albumin bound and free dyes.

The Oral Presentation

The following format can be used as a guide for your oral presentation. The presentation should last no more than five minutes, so you must be organized and concise.

1. Title

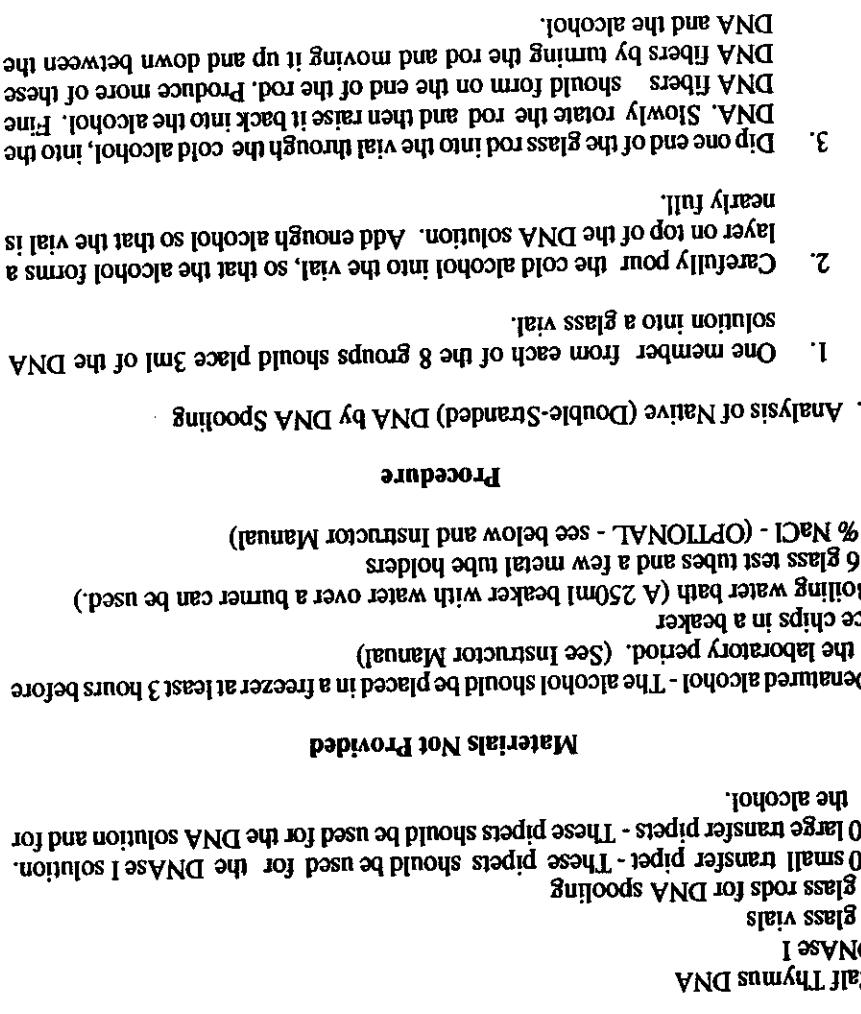
2. Introduction - A brief background of the topic is given in the introduction.

3. Objective - The goal of the experiment should be noted.

4. Results - The data collected is presented in the results section. For this presentation, you may use the actual gel, a diagram of the gel, or a figure or table containing the measurements that were made.

5. Discussion - You should emphasize the importance of the experiment, indicating the interpretations of the results.

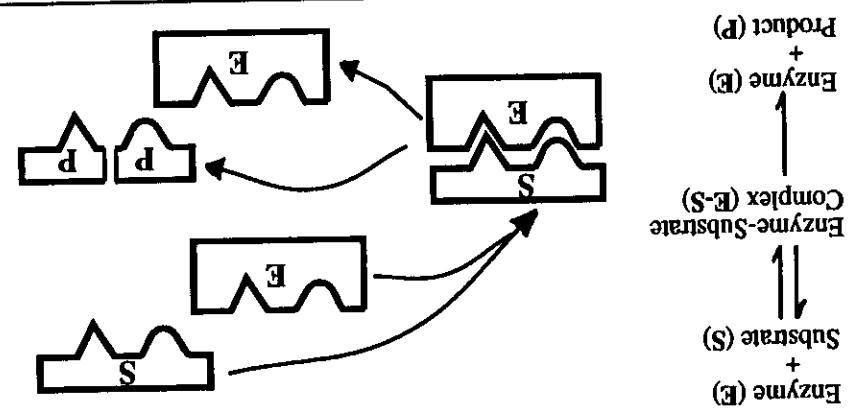
6. Conclusion - The conclusion should contain a restatement of the objective of the experiment and whether or not the objective was accomplished.



1. The term substrate refers to the compound that is acted upon by the enzyme.
2. The enzyme binds to the substrate to form an enzyme-substrate complex. This interaction is responsible for the specificity of enzyme action since only those compounds that "fit" into the substrate binding site can be acted upon by the enzyme. The binding of the substrate to the enzyme also serves to stabilize the enzyme.
3. The enzyme is not destroyed during the reaction but rather is set free after the formation of the end product. Thus, the liberated enzyme is available to catalyze in such a way that the conversion of substrate to product is facilitated.

Figure 1-1. Enzyme Action.

Three important features of enzyme reactions can be seen in the diagram shown in Figure 1-1.



Catalysis are agents that speed up chemical processes and the catalysts produced by living cells are called enzymes. Enzymes are proteins and each cell produces hundreds of them. Enzymes accelerate the velocity of virtually all reactions that occur in biological systems including those involved in breakdown, synthesis and chemical transfers. In so doing, they are responsible for performing essentially all the changes associated with life processes. The general expression frequently used to describe an enzyme reaction is shown in Figure 1-1.

Experiment B4-2. Extraction and Analysis of an Enzyme from Wheat

Background Information

Macromolecules such as DNA, RNA, and protein are not soluble in alcohol solutions, and precipitate (come out of solution) upon addition of alcohol. In general, globular proteins and RNA form fine, non-fibrillar precipitates in alcohol solutions, and precipitate (come out of solution) upon addition of alcohol. In contrast, the rod-like DNA molecules precipitate in alcohol as long fibers that can be spooled onto a glass rod. The ability of DNA to form fibers in alcohol depends on the physical properties of the DNA molecules. For example, DNA that has been broken into small pieces by DNase I digestion will not form fibers, nor will single-stranded DNA that has been prepared by heat denaturation of the DNA double-helix. These properties of DNA will be illustrated in today's experiments.

Objective To study the effects of DNase I and denaturation on the structure of DNA.

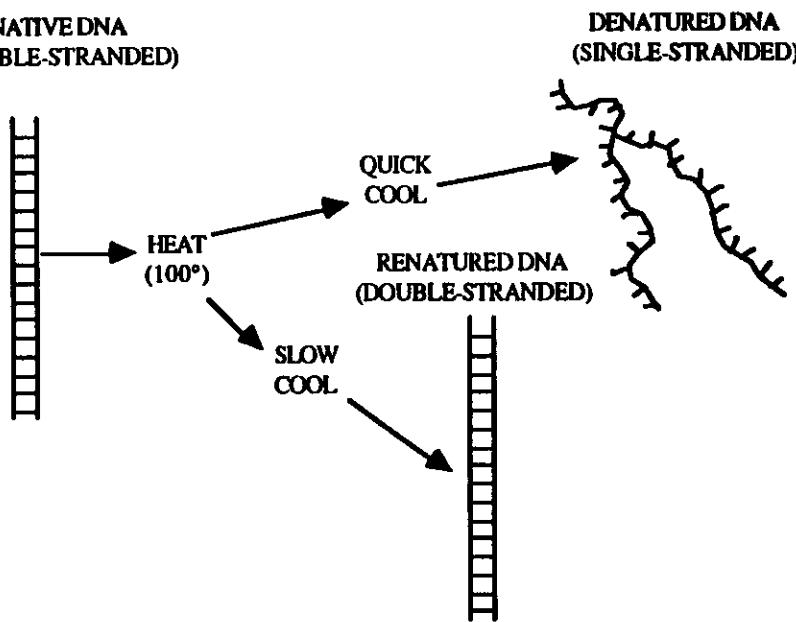
A. ENZYMES

Wheat

Background Information

A DNA molecule is composed of two polynucleotide chains that are coiled around each other to form a rigid double-helix (see page 4). The double-helical structure of DNA is very stable at room temperatures because the hydrogen and hydrophobic bonds between the stacked bases hold the two polynucleotide chains together. However, if a solution of DNA is heated to a critical temperature, these bonds are broken and the two polynucleotide strands separate by a process called denaturation. DNA denaturation is accompanied by a decrease in the viscosity (thickness) of the solution because single-stranded DNA molecules form flexible coiled structures that no longer retain the rigid native structure of the DNA double-helix (Figure 1-2). If the DNA is cooled rapidly, the molecules will remain as single stranded polynucleotides. However, if the solution is cooled very slowly, restoration of the DNA helix will occur. The reassembly of the two separated polynucleotide strands is called renaturation.

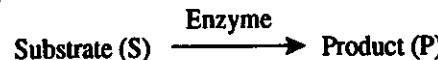
Figure 1-2. Denaturation of DNA.



combine with more substrate to produce more product. This feature makes enzyme molecules exceedingly efficient in catalysis and explains how very small quantities of enzymes are sufficient for cellular processes.

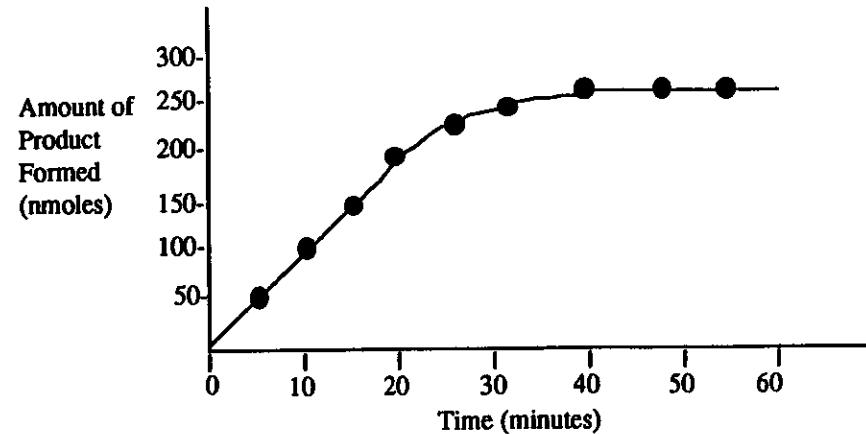
B. MEASUREMENT OF THE RATE OF ENZYME REACTIONS

An enzyme catalyzed reaction can be represented in a simplified form as follows:



The velocity or rate of the reaction can be determined by measuring the decrease in substrate concentration with time or, more commonly, by measuring the rate of appearance of product as shown in Figure 1-2. During the early part of the reaction illustrated in the figure, the amount of product formed increases linearly with time. However, in the latter part of the reaction (20 minutes to 40 minutes), the rate of product appearance diminishes, and at 40 minutes, product is no longer formed. A number of reasons can account for the decline in reaction rate with time including the depletion of substrate or the breakdown of the enzyme. In any case, in order to describe this reaction, its velocity must be determined during an early time interval when the amount of product is increasing in a linear manner. The rate measured during this time is the slope of a straight line and is called the initial velocity of the reaction (V_0). The V_0 in the example shown in the illustration is 10 nanomoles (nmoles*) of product formed per minute.

Figure 1-2. Measurement of the Rate of an Enzyme-Catalyzed Reaction.



The initial velocity, (V_0), is the slope of the linear portion of the curve. Therefore, in the example, $V_0 = \frac{\text{nmoles at time 2} - \text{nmoles at time 1}}{\text{time 2} - \text{time 1}} = \frac{100-50}{10-5} = \frac{50}{5} = 10 \text{ nmoles/min}$

*A mole is the molecular weight of a substance expressed in grams. Thus, one nanomole (n mole) is the molecular weight of a substance expressed in ng (1ng=10g⁻³). A one molar (1M) solution contains one mole of solute per liter of solution. Thus, a one nanomolar (1nM) solution contains one n mole of solute per liter.

The activity of enzymes is also greatly influenced by acidity or alkalinity. Enzymes act on alkalinity generally causes denaturation and inactivation of enzymes, just as high temperature causes heat denaturation. Most enzymes in plants and animals operate effectively at neutral pH. However, the pH optimum of the enzyme that you will study today is 4.5. In fact, the name of the enzyme, acid phosphatase, is derived in part from its low pH optimum.

pH.

Since enzymes are proteins, they are usually denatured and inactivated by temperatures above 50–70°C. On the other hand, increased temperature also speeds up chemical reactions. With a typical enzyme, the pre-denaturation effect of increased temperature up to about 45°C is to increase the enzyme catalyzed reaction rate. Above 45°C, thermal denaturation becomes increasingly important and destroys the catalytic function of the enzyme. At some temperature, a maximum reaction rate called the temperature optimum is observed, and this temperature is usually in the range found in cells (20°C–40°C).

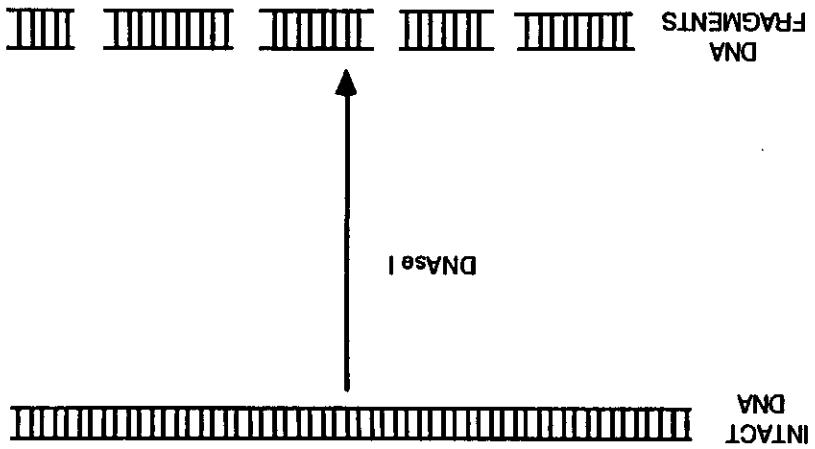


Figure 1-1. Breakdown of DNA by DNase I.

DNAs are extremely long molecule that is very thin, yet quite rigid. The isolation of intact DNA molecules from a cell is difficult because of the relative ease with which these long rod-like molecules can be broken. Even the injection of a solution of DNA through the needle of a hypodermic syringe can cause extensive breakdown of DNA molecules. DNA can also be broken down by enzymes called deoxyribonucleases. Deoxyribonuclease I (DNase I), an enzyme isolated from the mamalian pancreas, will be used in today's experiment. This enzyme breaks the phosphodiester bonds that connect the nucleotide units in DNA, degrading long DNA molecules to a mixture of small nucleotide chains, as illustrated in Figure 1-1.

Experiment (B-1). Properties of DNA

Background Information

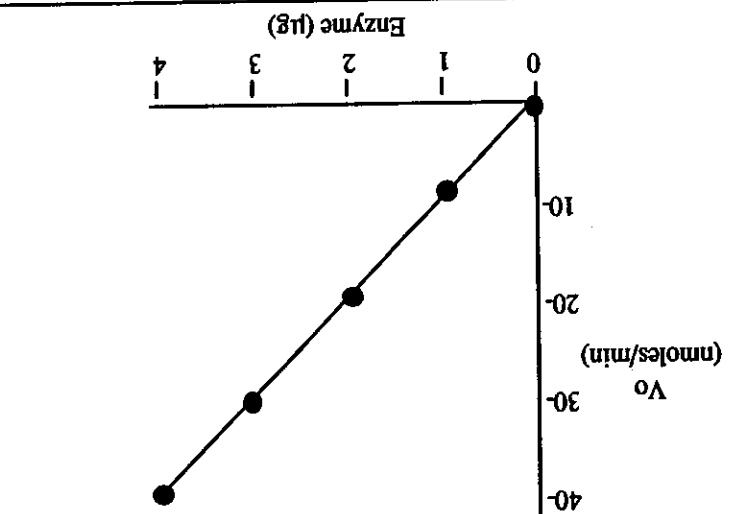
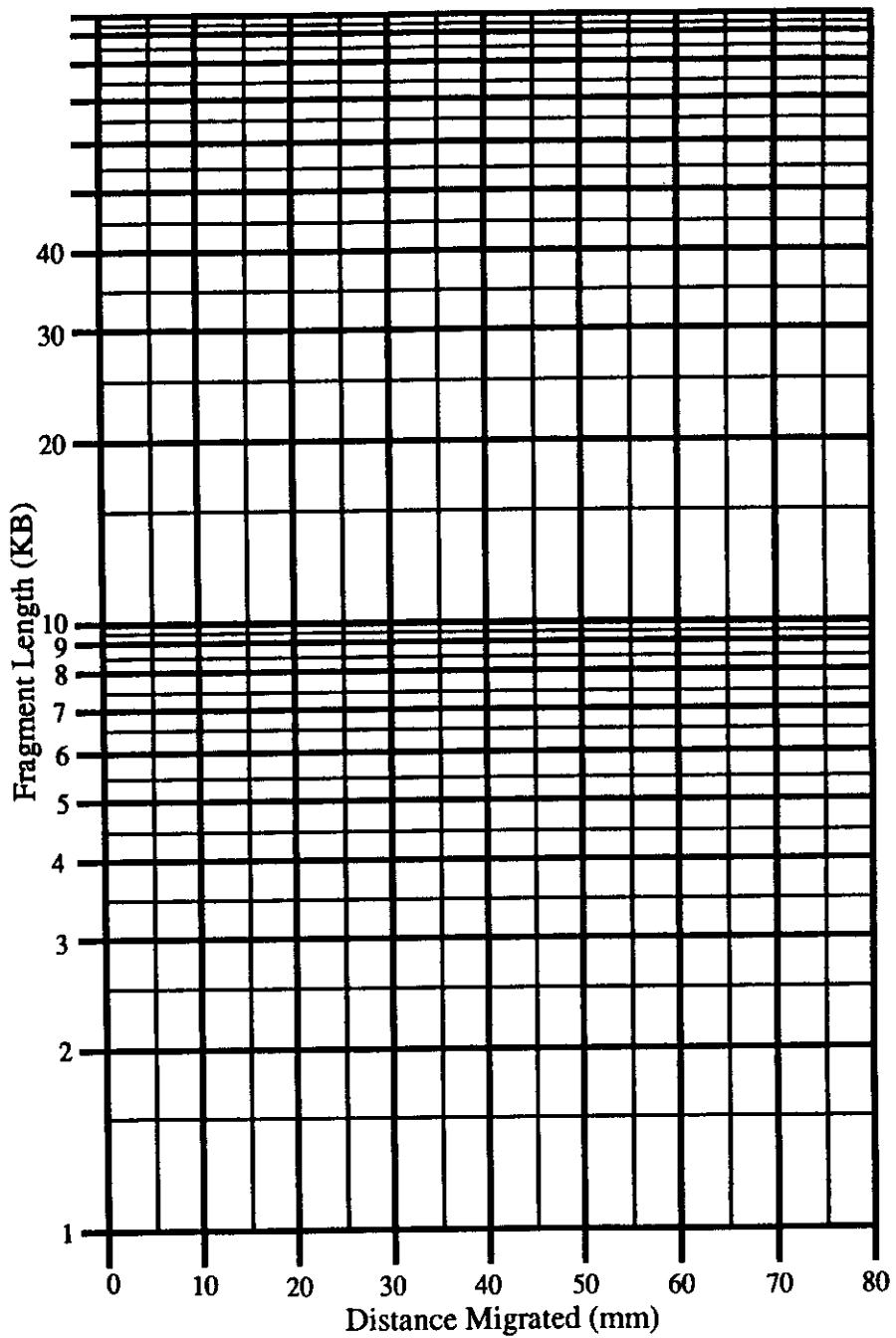


Figure 1-3. Initial Velocity and Enzyme Concentration.

The V_0 is proportional to the amount of active enzyme molecules as shown in Figure 1-3. This feature is important for it enables one to determine the amount of an enzyme in an unknown sample. For example, if 1 μg of a pure enzyme gives a V_0 of 10 nmoles of product/min, a cell extract that yields a V_0 of 20 nmoles produces/min contains 2 μg of active enzyme.

C. FACTORS THAT INFLUENCE THE RATE OF AN ENZYME CATALYZED REACTION

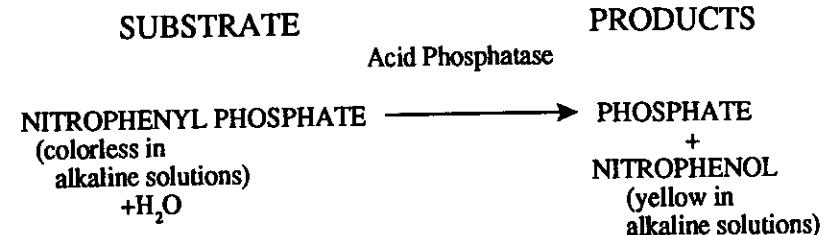


D. WHEAT GERM ACID PHOSPHATASE

The wheat kernel consists of three parts: (1) the embryo or germ that produces the new plant (2) the starchy endosperm which serves as a food source for the embryo and (3) covering layers which protect the grain. The endosperm is the raw material for flour production, while the wheat germ is used in food products as a source of vitamins. Wheat germ is also frequently used in the molecular biology laboratory as a source of plant cell proteins and nucleic acids. In today's laboratory, you will analyze wheat germ acid phosphatase.

The study of enzymes frequently begins with the extraction of these proteins from tissue. Enzyme extraction procedures usually require a method that destroys the integrity of the cell. The broken cells then release their molecular constituents including enzymes. One method that can be used to lyse (break open) cells involves treating tissue with a detergent that breaks or dissolves cell membranes. In the procedure described below, you will use an enzyme extraction buffer that contains the detergent NP-40 in order to prepare an enzyme extract from wheat germ containing acid phosphatase.

Acid phosphatase catalyzes the removal of phosphate groups from macromolecules and smaller molecules that are stored in the wheat seed. The free phosphate is then used by the growing embryo. In the exercise described below, you will measure the velocity of the reaction catalyzed by purified acid phosphatase and by the acid phosphatase that is found in the wheat germ extract that you prepare. A synthetic phosphatase substrate called nitrophenyl phosphate will be used in the experiment. Nitrophenyl phosphate is colorless but is broken down to free phosphate and nitrophenol as shown below. Nitrophenol is yellow in alkaline solution so that the appearance of yellow color is indicative of the amount of product formed in the reaction.



Objectives To prepare an enzyme extract from wheat germ, and then to determine the amount of acid phosphatase in the extract.

Laboratory Schedule

This experiment was designed for 8 groups of students. The exercise can be completed within a 1-1.5 hour laboratory period.

Materiials Provided

- *Nitrophenol-Standards - The 6 standards contain 0, 25, 50, 100, 200, and 400 nmoles of nitrophenol per 2ml of KOH.
- *Acid Phosphatase Substrate Solution - This solution contains nitrophenyl phosphate (1mM) dissolved in an acetate buffer which has a pH of 4.5.
- Acid Phosphatase - The concentration of the purified enzyme provided is 5U/g/ml.
- Wheat Germ - Upon completion of the experiment, return the wheat germ to the freezer.
- *Enzyme Extraction Buffer - The buffer contains the detergent NP-40 which will facilitate the lysis of the wheat germ cells.
- *15% KOH - Handle this base with caution.
- Small Transfer Pipets
- 16 Glass Tubes (25ml)
- Cheese Cloth - The cloth will not be needed if a centrifuge is available.
- *Prepared as described in Appendix 2 of the Instructor Manual.
- Materiials Not Provided

mm migrated

lanes 1, 5 -
lanes 2, 6 -
lanes 3, 7 -
lanes 4, 8 -

Data Analyses and Study Questions

4. Design the gels and place your gel over a light source. Measure the distance of all DNA bands (in mm) from the sample origin. Record these values below.
- *Nitrophenol-Standards - The 6 standards contain 0, 25, 50, 100, 200, and 400 nmoles of nitrophenol per 2ml of KOH.
- *Acid Phosphatase Substrate Solution - This solution contains nitrophenyl phosphate (1mM) dissolved in an acetate buffer which has a pH of 4.5.
- Acid Phosphatase - The concentration of the purified enzyme provided is 5U/g/ml.
- Wheat Germ - Upon completion of the experiment, return the wheat germ to the freezer.
- *Enzyme Extraction Buffer - The buffer contains the detergent NP-40 which will facilitate the lysis of the wheat germ cells.
- *15% KOH - Handle this base with caution.
- Small Transfer Pipets
- 16 Glass Tubes (25ml)
- Cheese Cloth - The cloth will not be needed if a centrifuge is available.
- *Prepared as described in Appendix 2 of the Instructor Manual.
- Materiials Not Provided

1. On the semilog paper provided on the following page, plot the distance migrated by the DNA bands from phage lambda (lanes 2 and 6) as a function of the lengths of these fragments. The lengths of the fragments are 23.1 KB, 9.4 KB, 6.6 KB, 4.4 KB, 2.3 KB, and 2.0 KB (see Figure 2).
2. Determine the length, in KB, of plasmid PUC18 by the method described in Figure 1.
3. Estimate the length of the major satellite band from cow (lanes 3, 4, 7, 8).
4. The length of DNA helix occupied by one nucleotide pair is 3.4 Å (angstrom).
5. A human has 10^{14} cells and each human cell has about 6.4×10^9 nucleotide pairs of DNA. What is the length of double helix that could be formed from this amount of DNA in a human individual?

- *Nitrophenol-Standards - The 6 standards contain 0, 25, 50, 100, 200, and 400 nmoles of nitrophenol per 2ml of KOH.
- *Acid Phosphatase Substrate Solution - This solution contains nitrophenyl phosphate (1mM) dissolved in an acetate buffer which has a pH of 4.5.
- Acid Phosphatase - The concentration of the purified enzyme provided is 5U/g/ml.
- Wheat Germ - Upon completion of the experiment, return the wheat germ to the freezer.
- *Enzyme Extraction Buffer - The buffer contains the detergent NP-40 which will facilitate the lysis of the wheat germ cells.
- *15% KOH - Handle this base with caution.
- Small Transfer Pipets
- 16 Glass Tubes (25ml)
- Cheese Cloth - The cloth will not be needed if a centrifuge is available.
- *Prepared as described in Appendix 2 of the Instructor Manual.
- Materiials Not Provided

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- Acid Phosphatase - The concentration of the purified enzyme provided is 5U/g/ml.
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- *15% KOH - Handle this base with caution.
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- 16 Glass Tubes (25ml)
- Cheese Cloth - The cloth will not be needed if a centrifuge is available.
- *Prepared as described in Appendix 2 of the Instructor Manual.
- Materiials Not Provided

Objectives

To compare the electrophoretic patterns of restriction digests of a plasmid (pUC18), phage lambda DNA, and cow DNA from thymus and kidney.

Materials Provided

Plasmid DNA - EcoR1 cut: The plasmid is known as pUC18 (see Figure 3)

Phage Lambda DNA - Hind III cut (see Figure 2)

Calf Thymus DNA - EcoR1 cut

Calf Kidney DNA - EcoR1 cut

Materials Needed but not Provided

Agarose, electrophoresis buffer, and gel stain

Procedure

The procedures for the preparation, electrophoresis and staining of the agarose gels are described in detail in the first part of this manual and are briefly outlined below. This experiment is designed so that the samples of two students will be analyzed on one agarose gel. If the students work in pairs, four students will share one gel. The samples of each student (or student pair) will be electrophoresed on four gel lanes.

1. Prepare the agarose gels as described in the first part of this manual. In this experiment, 1.2% gels will be used.
2. Load 15 µl of each sample into the wells as indicated below.

Sample Well Number	Sample
1	Plasmid DNA - EcoR1 cut
2	Phage Lambda DNA - Hind III cut
3	Calf Thymus DNA - EcoR1 cut
4	Calf Kidney DNA - EcoR1 cut
5	Plasmid DNA - EcoR1 cut
6	Phage Lambda DNA - Hind III cut
7	Calf Thymus DNA - EcoR1 cut
8	Calf Kidney DNA - EcoR1 cut

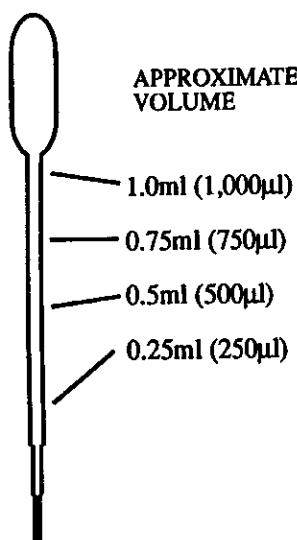
3. Electrophorese until the bromophenol blue (blue dye) in the DNA samples has migrated to within 1 mm of the positive electrode end of the gel. At 170 volts, this should take about 50 minutes. Remove the gels from the unit and stain them with methylene blue overnight in the refrigerator.

The Pipets

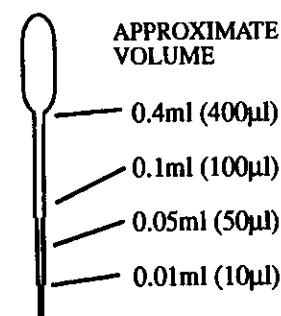
The basic tools you will have available for measuring liquids are the macropipets and two types of transfer pipets. The macropipets are calibrated in mls and should be used for measuring volumes greater than 2 mls. A diagram of the transfer pipets is given below. Shown in the figure are the approximate volumes of liquid dispensed if the pipets are filled to the indicated levels. The large transfer pipets should be used for measuring volumes between 0.5-1.0ml and the small transfer pipets for volumes less than 0.5 ml. Thus, to transfer 0.1ml (100µl) from tube A to tube B, place the tip of a small transfer pipet in tube A, depress the bulb slightly with your thumb and index finger, slowly relax your index finger until the solution has filled the pipet to the 100 µl level, transfer the pipet to tube B and depress the bulb. A separate pipet should be used by the entire class for dispensing each solution provided with this Program. Students should practice using these pipets prior to beginning the experimental exercises.

Transfer Pipets

LARGE TYPE

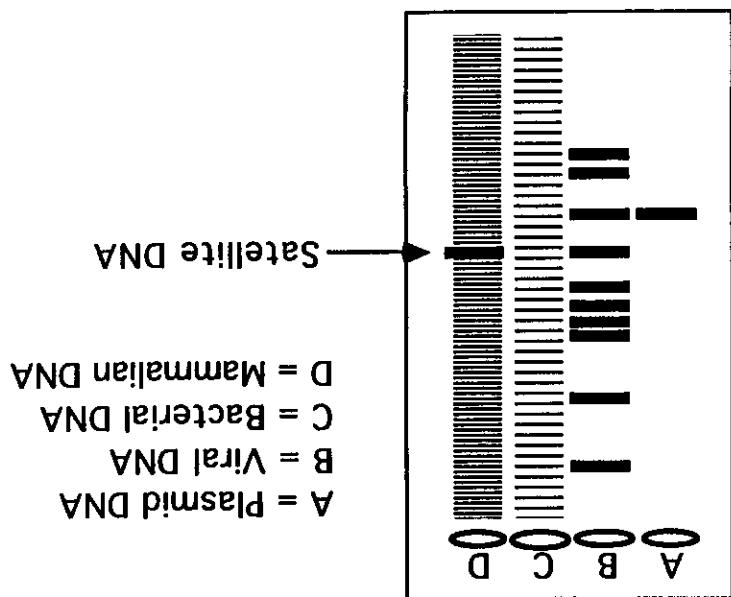


SMALL TYPE



The bacterial genome, each of the gel lanes, since there is essentially no repetitive DNA in appear as smears on the gel lanes. Since there is no repetitive DNA in the bacterial genome, each of the 1,000 bands will be of the same intensity. Thus, the smear on the gel lane will appear uniform. However, fragments derived from highly repeated sequences in the mammalian genome will form discrete bands which are superimposed on the background smear of the large number of different size fragments. Thus, highly repeated sequences like satellite DNA can be detected readily by standard techniques in the eukaryotic genome that can be detected readily by standard electrophoretic procedures.

Figure 4. Analyses of Genome Complexity by Electrophoresis.



The four DNA preparations were digested with ECOR I prior to this electrophoretic separation.

- Figure 4. Analysis of Genome Complexity by Electrophoresis.**

D = Mammalian DNA
C = Bacterial DNA
B = Viral DNA
A = Plasmid DNA

Satellite DNA

Table 2. Organization of Genomes

Genome Type		
<u>Plasmid, Viral, and Prokaryotic Genomes</u>	<u>Eukaryotic Genomes</u>	
Genes		
Average Length	~ 1,000 bp	~5,000 bp
Introns	Rare	Common
Copies per Genome	Usually one	Often more than one
Spaces Between Genes		
	Usually small (less than 300 bp)	Usually large
Repeated Nongenic Sequences (e.g. DNA satellites)		

C. Description of this laboratory exercise

Figure 4 shows the results of an experiment similar to the one that you will perform in this laboratory. In the analysis, DNA from four sources was digested with EcoR1 and the fragments were then separated according to size by electrophoresis on an agarose gel. The DNA was obtained from A) a 4KB plasmid B) a 40 KB virus C) a bacterium with a genome size of 4×10^3 KB and D) a mammal with a genome size of 4×10^6 KB. The recognition site for EcoR1 is a specific sequence of 6 nucleotides. This recognition site is found, on average, once in every 4KB of genomic DNA. Consequently, one restriction fragment is derived from the genome of the plasmid, 10 from the genome of the virus, 10^3 from the bacterial genome, and 10^6 from the mammalian DNA. The small numbers of individual bands can clearly be distinguished in the digests of the plasmid and viral DNA's. However, the large number of fragments from the bacterial and especially mammalian genomes will

7. At the times indicated below, remove 1ml of the solutions in tubes A* and B* and place them in the corresponding tubes.

<u>Time</u>	<u>Tube Number</u>
0 minutes	A1 and B1 - see above
2.5 minutes	A2 and B2
5 minutes	A3 and B3
10 minutes	A4 and B4
15 minutes	A5 and B5
20 minutes	A6 and B6

The KOH in the tubes serves a dual purpose. First, it will stop the reaction because acid phosphatase will be catalytically inactive at alkaline pH. Secondly, the KOH will cause one of the products of the reaction (nitrophenol) to turn yellow, and the intensity of the yellow color is proportional to the amount of nitrophenol in the well.

III. Measurement of the Product (Nitrophenol) of the Reaction

1. In order to determine the amount of nitrophenol produced in the reactions, you will compare the intensity of the colored reaction products to the intensities of known amounts of nitrophenol. The six nitrophenol standards that will be distributed by your instructor contain the following amounts of nitrophenol dissolved in 2ml of KOH.

1. 0
2. 25 nmoles
3. 50 nmoles
4. 100 nmoles
5. 200 nmoles
6. 400 nmoles

Number the remaining 6 tubes in your rack #1-6C. Using a transfer pipet, place 2ml of these standards into the corresponding tubes.

2. The amount of nitrophenol can be measured by visual inspection and/or by using a colorimeter such as the Bausch and Lomb Spectronic 20.

A. VISUAL DETERMINATION

1. Hold each tube over a white sheet of plain paper and examine the intensity of the yellow color produced by the known amounts of nitrophenol (tubes numbered C1-C6).

Satellite DNA is not found in bacteria but is found in the genomes of nearly all eukaryotic organisms. In some vertebrates (e.g., chickens and humans), satellite sequences occur in low concentrations. In other species (e.g., kangaroos and meadowlarks), these sequences can make up as much as 60% of the genome. The function of satellite DNA is not known. However, it is known that most satellite sequences are found in discrete regions along the chromosomes. For example, many satellites are found in the centromeres of each chromosome. Centromeres are the sites of attachment of the chromosomes to the mitotic spindle and satellite DNA may play some role in the attachment of the mitotic spindle. Centromeres are also found in the nucleolus of the cell nucleus. Each differentiated cell in a multicellular organism contains the same number of chromosomes and the chromosome set appears identical in all cell types. As a general rule, the genome is also identical in all different cells of the body.

Thus, the genes that code for α and β globin in all different cells of the body are found in the same form in all cells including immature erythrocytes that produce hemoglobin and nonerythroid cells that do not. Likewise, the order of nucleotides or nucleotide sequence of satellite DNA is invariant in all cell types and this feature of the genome will be illustrated in today's laboratory exercise.

The genome of a higher eukaryote contains about 1,000 times more DNA than the genome of a bacterium (see Table 1). However, the actual number of different genes in the eukaryotic genome is only about 10–20 times more than in the bacterial genome. Differences in the organization of the prokaryotic and eukaryotic genomes are responsible for the excess DNA that is observed in the eukaryotic nucleus. These differences are outlined in Table 2 and discussed below.

First, most eukaryotic genes contain introns which are inserted from species that are precursors to mRNAs. These genes give rise to RNA code for mRNA or protein. During transcription, these genes are removed from the precursor and destroyed, and the remaining segments of the precursor are spliced together to form mRNA which can then be translated into protein. Genes in bacteria, viruses, and plasmids rarely contain introns. Thus, these genes are shorter than those seen in eukaryotes. Second, many proteins coding genes are found in multiple copies within the eukaryotic genome. For example, there are multiple copies of growth hormone genes in humans. In contrast, nearly all genes in bacteria, viruses, and plasmids are present in only one copy per genome. Third, eukaryotic genes, unlike genes in the bacterial genome, are frequently separated by long stretches of noncoding DNA. Fourth, some of the noncoding DNA is compressed or stretched out of the genome. Fifth, some of the noncoding DNA is composed of repeating units called repeats. Sixth, the eukaryotic genome contains many genes that are not expressed at all.

Eukaryotic Genomes

- | Eukaryotic Genomes | | | | | |
|--------------------|--------------------------|----------------------|--|----------------|--------|
| Tube | Reaction Tube Numbers of | Nitrophenol (mMoles) | Standards that Yield equivalent (mMoles) | Time (minutes) | Number |
| A1 | 0 | CI | Yield Color | CI-C6 | |
| A2 | 2.5 | CI | | | |
| A3 | 5 | | | | |
| A4 | 10 | | | | |
| A5 | 15 | | | | |
| A6 | 20 | | | | |
| B1 | 0 | CI | | | |
| B2 | 2.5 | | | | |
| B3 | 5 | | | | |
| B4 | 10 | | | | |
| B5 | 15 | | | | |
| B6 | 20 | | | | |

Plasmid Genomes

Plasmids are small circular DNA molecules that exist apart from the chromosomes in most bacterial species. Under normal circumstances, plasmids are not essential for survival of the host bacteria. However, many plasmids contain genes that enable bacteria to survive and to prosper in certain environments. For example, some plasmids carry one or more genes that confer resistance to antibiotics. A bacterial cell containing such a plasmid can live and multiply in the presence of the drug. Indeed, antibiotic-resistant *E. coli* isolated in many parts of the world contain plasmids that carry the genetic information for protein products that interfere with the action of many different antibiotics.

Plasmids, like viruses, contain a specific nucleotide sequence that serves as a regulatory signal for the initiation of replication. This sequence is called the origin of replication. Plasmids, like viruses, also contain genes that are necessary for the survival of the plasmid in the host cell. Plasmid encoded antibiotic resistance genes provide examples of these coding sequences. Unlike viruses, however, plasmids do not make a protein coat and thus cannot readily move from cell to cell.

Plasmids that are used in recombinant DNA research are typically small circular DNA molecules and you will determine the size of one such plasmid in this exercise. A diagram of this plasmid is shown in Figure 3. The plasmid, called pUC18, contains an origin of replication and an antibiotic resistance gene. The plasmid has been linearized by digestion with the restriction enzyme EcoR1 and you will determine the size of the linear form of this DNA molecule in today's laboratory.

Bacterial Genomes

The genome of a typical bacterium such as *E. coli* consists of a single circular DNA molecule that is associated with few proteins. The *E. coli* genome contains approximately 4.5 million base-pairs of DNA which represents about 4,000 genes. These genes are, on average, 1,000 base-pairs in length. Bacterial genes are closely spaced around the genome and there is little DNA between them. Nearly all bacterial genes are unique in that each is found in only one copy per genome. The economy in the genome permits rapid rates of genome replication which can be as short as 20 minutes in some bacteria including *E. coli*. Rapid replication enables bacteria to divide quickly and this feature permits bacterial species to occupy and to saturate ecological niches in short periods of time.

- From the graph, calculate the initial velocities (V_0) for the two reactions.

Reaction A1-A6 V_0 = _____ nmoles nitrophenol/minute

Reaction B1-B6 V_0 = _____ nmoles nitrophenol/minute

B. SPECTROPHOTOMETRIC DETERMINATION

You may wish to use a colorimeter to determine the intensity of the colored product. Prior to performing this procedure, the student should be familiar with the basic operation of the colorimeter that will be used.

- Allow the colorimeter to warm up at least 5 minutes prior to taking the readings. During the warm up period, add 10ml of water to each of your 18 tubes and carefully mix the contents.
- Set the wavelength to 410nm and adjust the dial so that it reads 0% transmittance.
- To blank the spectrophotometer, transfer the contents of tube#1C to a cuvette, insert the cuvette into the cuvette holder, close the cover and adjust the needle to 100% transmittance. With this solution as the blank, read the absorbance of each standard (tube number 2C-6C) as described above and record these data in the table below.
- Read the absorbance (A) of each time point in your reactions (tube numbers A1-A6 and B1-B6) and record these data below.

Absorbance (A) at 410nm

Tube <u>Number</u>	Tube <u>Number</u>	Tube <u>Number</u>
A1	B1	C1 0.0
A2	B2	C2
A3	B3	C3
A4	B4	C4
A5	B5	C5
A6	B6	C6

Amp^r = Ampicillin - Resistance Gene

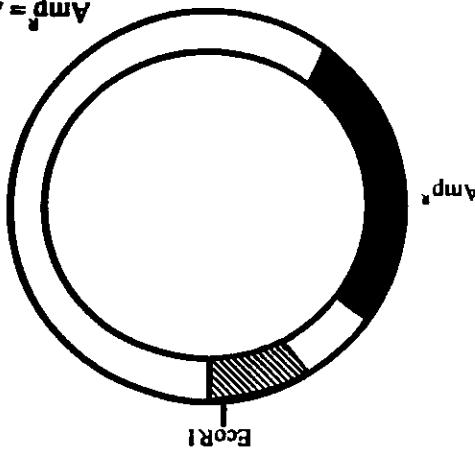


Figure 3. Plasmid pUC18

Position of Hind III Restriction Sites

Size of Hind III Restriction Fragments

Arrows indicate the position of restriction sites for Hind III. Sites are in base-pairs from the left end of the phage DNA.

B. Restriction Map of DNA - Hind III

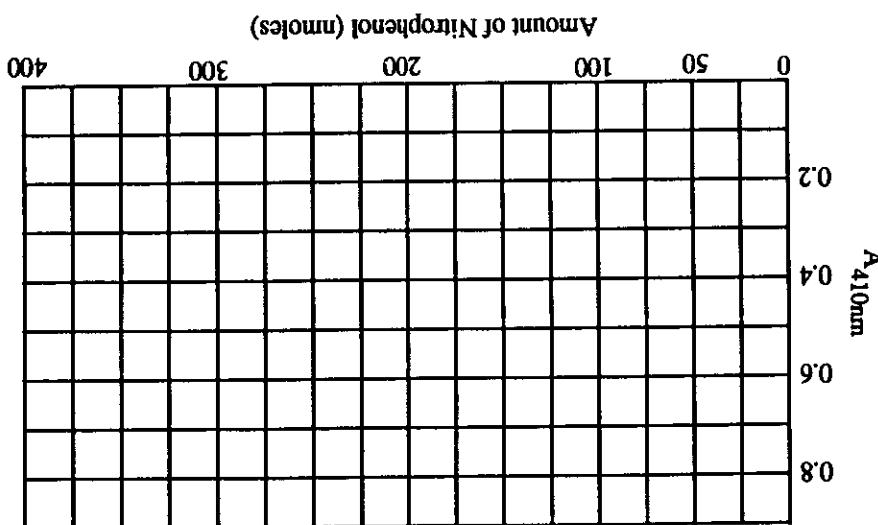
A. Genetic Map of Phage Lambda

The figure shows a horizontal genetic map of Phage Lambda. The map is labeled with positions in Kilobase-pairs (Kbp) from 0 to 50. Below the map, several genetic features are indicated by arrows pointing to specific locations:

- Attachment:** An arrow pointing to approximately 15 Kbp.
- Recombination:** An arrow pointing to approximately 25 Kbp.
- Integration:** An arrow pointing to approximately 30 Kbp.
- Excision:** An arrow pointing to approximately 35 Kbp.
- Tail Proteins:** An arrow pointing to approximately 40 Kbp.
- Head Proteins:** An arrow pointing to approximately 45 Kbp.
- Lysis:** An arrow pointing to approximately 50 Kbp.
- Control:** A bracketed region spanning from approximately 10 Kbp to 20 Kbp, containing the *attP*, *cro*, *cob*, *cro*, and *q_{pro}* genes.

Figure 2. Genetic and Restriction Map of Phage Lambda DNA.

11. Prepare the standard curve for the assay using the graph below. Plot the absorbencies for each standard (tube numbers C1-C6) on the Y axis as a function of the amount of nitrophenol in the standards on the X axis.



2. Using this standard curve, determine the number of nitrophenol produced in each tube of your assay, (A1-A6 and B1-B6). Record these data below, and then plot the number of nitrophenol produced against time in the graph.

Detailed description: This is a scatter plot with a grid. The y-axis is labeled 'Nitrophenol Produced (nmoles)' and has major tick marks at 0, 100, 200, 300, and 400. The x-axis is labeled 'Time (minutes)' and has major tick marks at 0, 2.5, 5, 10, 15, and 20. Six data series are plotted, each representing a sample labeled A1-O through A6. Each series shows a positive linear correlation between time and nitrophenol produced.

Sample	Time (minutes)	Nitrophenol Produced (nmoles)
A1-O	0	0
A1-O	10	200
A2-	0	0
A2-	10	200
A3-	0	0
A3-	10	200
A4-	0	0
A4-	10	200
A5-	0	0
A5-	10	200
A6-	0	0
A6-	10	200

Data Analysis

Viral Genomes

Viruses are noncellular organisms that can reproduce only within host cells. Essentially all cellular organisms can be infected with viruses. Thus, there are viruses that attack plants, animals, and bacteria. Viruses that infect bacteria are called bacteriophages or simply phages. All viruses contain a genome which is surrounded and protected by a protein coat. The viral genome contains genes that code for these coat proteins. Other genes are also found in the genome that are necessary for the viral life cycle. In addition, the genome contains regulatory signals for viral transcription and replication. However, viral genomes vary considerably in different viral types. Thus, the genome can be composed of either DNA or RNA, can be single-stranded or double-stranded, and can be circular or linear.

Bacteriophage lambda, which infects *E. coli*, is probably the best understood of the double-stranded DNA phages. The protein component of phage lambda consists of a protective coat which forms the tail assembly and the outer shell of the head. A single molecule of double-stranded DNA is located in the core of the phage head. The DNA molecule contains 48,502 base-pairs (molecular weight- 3×10^7) that codes for approximately 50 different phage proteins. The sequence of nucleotides along the entire lambda genome is known and the nucleotide sequences that comprise the major control regions for transcription and replication have been identified.

Figure 2 shows a genetic map of the phage lambda genome. The genes of the phage are clustered according to their function and the DNA may be divided into three regions. The left-hand region includes all the genes (A through J) whose products are necessary to produce phage head and tail proteins and to package the DNA into the virus. The central region contains elements involved in integration of the DNA into the *E. coli* chromosome. The remaining portion of the genome includes the major control region for transcription and replication, and the genes necessary for cell lysis. In order to study these regions, a method is needed to cut the genome at defined sites. Restriction endonucleases are used for this purpose and these enzymes are discussed in Part A of this manual. In this exercise, the lambda DNA that you will use was obtained by digestion of the lambda genome with the restriction endonuclease Hind III. The positions of the restriction sites for restriction nuclease Hind III are given at the bottom of Figure 2. The lengths of the DNA fragments produced when lambda DNA is cleaved with this enzyme are also shown.

- From the above graph, calculate the initial velocities (V_0) for the two reactions.

Reaction A1-A6 $V_0 = \underline{\hspace{2cm}}$ nmoles nitrophenol/minute

Reaction B1-B6 $V_0 = \underline{\hspace{2cm}}$ nmoles nitrophenol/minute

Study Questions

- Given that you added (or should have added) about 5 μ g of purified acid phosphatase to tube A, calculate the amount of acid phosphatase that was present in the 400 μ l of the wheat germ extract in the tube B.
- How much acid phosphatase is present in 1g of wheat germ?

NADH_+ = Nicotinamide-adenine dinucleotide
 NAD^+ = Nicotinamide-adenine dinucleotide
 LDH = Lactate Dehydrogenase

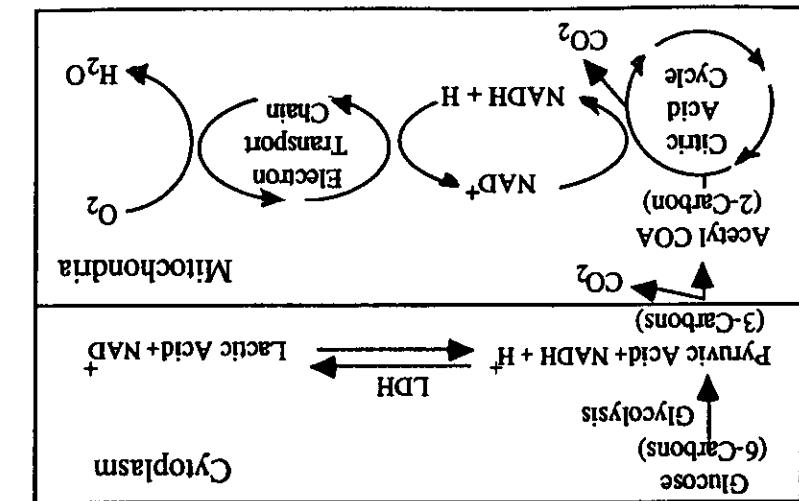


Figure 3-1. Summary of Respiratory Metabolism.

	Approximate Size of Haploid Genome	Number of Killobase-pairs (of DNA)	Genome Type	Chromosomes
DEPENDENT ENTITIES	(Requires true living organisms for growth and reproduction)	Plasmids	pBR322	4
		Viruses	Phage Lambda	50
		Human Immunodeficiency	Virus (Provirus)	10
TRUE LIVING ORGANISMS	Chloroplasts from mammals	16-19	Bacteria (E. coli)	1
	Mitochondria from mammals	120-200	Prokaryotic Cells	1
	Organelles	4,000	Eukaryotic Cells	16
	Chloroplasts from higher plants	120-200	Yeast (S. cerevisiae)	16
		14,000	Fruit Fly (D. melanogaster)	4
		170,000	Toad (X. laevis)	18
		3,000,000	Human (H. sapiens)	23
		5,000,000	Com (Z. mays)	10

Table 1. Genome Sizes

The basic pattern of glucose oxidation is outlined in Figure 3-1. Glucose is split into two molecules of pyruvic acid, each with three carbon atoms. These reactions occur in the cytoplasm by a process called glycolysis. In the presence of oxygen (aerobic conditions), pyruvic acid then enters the mitochondria, where it is completely degraded to CO_2 and water in the citric acid cycle and in the electron transport chain. During the course of this process, the electrons are given up to the carrier molecule NADH_+ . This is thereby reduced to NAD^+ . The electrons are liberated as CO_2 , while the hydrogen atoms (protons + electrons) are transferred primarily to the carrier molecule NAD^+ , which is thereby reduced to NADH_+ . The electrons are then transferred from the NADH_+ to a series of electron carriers that comprise the electron-transport chain. During the course of this chain, the electrons are given up to the carrier molecule NADH_+ . This is thereby reduced to NAD^+ . The electrons are then transferred to the terminal electron carrier, the electron acceptor, which is usually molecular oxygen (O_2). The electrons enter the electron-accepting protein complex, where they combine with molecular oxygen to form water.

A. BIOCHEMISTRY

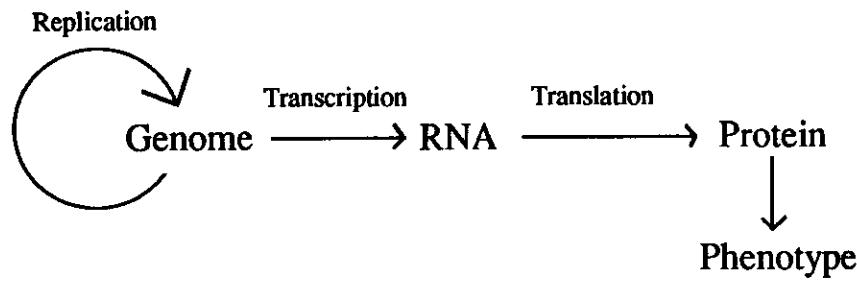
Background Information

Experiment 3 (803). Tissue-Specific Isoenzymes

from this calibration curve. In practice, DNA standards and unknown DNAs are electrophoresed on adjacent lanes of the same agarose gel. After electrophoresis, the positions of the standard and unknown DNA bands in the gel are determined and the size of the unknown calculated. The length of DNA is frequently given in base-pairs (BP) for small fragments and kilobase pairs (KB) for large ones. One kilobase-pair equals 1000 base-pairs. In this laboratory, you will use the procedure shown in Figure 1 to determine the size of DNA molecules.

B. The Genome

The genetic material of an organism is known as the genome. The genomes of all organisms direct two fundamental processes as diagrammed below. First, genes within the genome code for proteins and the proteins dictate phenotype by controlling cell structure and function. Second, the genome is self-replicating which provides genetic continuity through cell division and from one generation to the next.

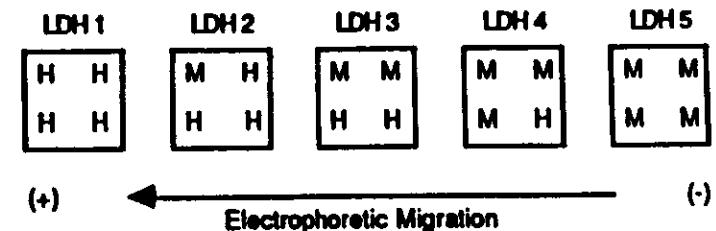


Cellular organisms are frequently classified into two basic types: prokaryotic and eukaryotic. The prokaryotic plan is seen in bacteria and blue green algae. The prokaryotic cell lacks a discrete nucleus and the genome usually consists of a single circular DNA molecule that is compacted within the cell interior. The eukaryotic plan is observed in true algae, fungi, protozoans, and the cells of higher animals and plants. The genome of the eukaryotic cell is found in the nucleus where the DNA is portioned into a number of chromosomes. Genomes are also found in some groups of noncellular entities. These entities include viruses, plasmids, and certain cytoplasmic organelles that are found in eukaryotes including chloroplasts and mitochondria. This group shares with true living organisms the property that one generation gives rise to the next. Their genomes also contain genes which code for certain proteins that are responsible for their phenotype. However, these entities are dependent on true living organism in that they can grow and reproduce only within a host prokaryotic or eukaryotic cell. Some examples of genome sizes is provided in Table 1 and a brief discussion of the genomes that you will study in this exercise is given below.

The final reaction of anaerobic (without oxygen) glycolysis is the conversion of pyruvate to lactic acid and this reaction is catalyzed by the enzyme lactate dehydrogenase (LDH). In skeletal muscle, where oxygen deprivation is common during exercise, the reaction is efficient and large amounts of lactate can be formed. (See Figure 3-1). In tissues that preferentially oxidize glucose aerobically to CO₂ and water such as cardiac muscle, the reaction is not efficient and pyruvate is preferentially converted to acetyl COA which enters the citric acid cycle. In order to understand the differences in efficiency of this reaction in skeletal and heart muscle, it is necessary to explore the structure of the LDH enzyme in different tissues of the body.

Isoenzymes are different molecular forms of the same enzyme and five major LDH isoenzymes are found in different vertebrate tissues. Each LDH molecule is composed of four polypeptide chains (each is a tetramer) but the subunit composition of the five LDH isoenzymes are different. There are two types of polypeptide chains in LDH called M (for skeletal muscle) and H (for heart muscle) which can be combined into the LDH tetramer in 5 different ways. Each different combination of subunits represents a distinct LDH isoenzyme as illustrated in Figure 3-2. Because the H polypeptide has more acidic amino acid residues than the M polypeptide, the electrophoretic mobilities of the LDH isoenzymes are: LDH 1 > LDH 2 > LDH 3 > LDH 4 > LDH 5.

Figure 3-2. LDH Isoenzymes.



The H and M polypeptides of LDH are encoded by different genes, and the two genes are expressed to different degrees in different tissues. For example, in heart muscle, the gene for the H subunit is more active than the gene for the M subunit. Thus, LDH isoenzyme 1 is the predominant form of the enzyme in cardiac muscle. The reverse is true in skeletal muscle where there is more M than H polypeptide produced, and hence, more of the isoenzyme 5 form of the enzyme. The efficiency of the conversion of pyruvate to lactate increases with the number of M chains. Therefore, the high concentration of LDH-5 (4 M subunits) in skeletal muscle rapidly converts pyruvate to lactate while the high concentration of LDH-1 (4 H subunits) in heart tissue favors conversion of pyruvate to acetyl COA which enters the citric acid cycle. These tissue-specific differences in LDH-isozymes can be readily detected by the localization of LDH activity in an agarose gel after electrophoresis of tissue extracts as shown in Figure 3-3.

Experiment 1001. Anatomy and Evolution of the Genome.

A. Determining the Length of DNA Molecules

A first step in the analysis of a DNA molecule in the molecular biology laboratory frequently involves the determination of its length in nucleotide pairs. Electro- phoresis in agarose gels has proven to be an extremely useful tool for this purpose as the length of a given DNA fragment can be determined by comparing its electrophoretic mobility on agarose gels with DNA markers of known lengths. The smaller a DNA fragment, the more rapidly it moves during electrophoresis. As shown in Figure 1, a linear relationship is obtained if the logarithms of the sizes (in base-pair units) of the DNA fragments are plotted against their respective electro- phoretic mobilities. The length of an unknown DNA fragment is then estimated

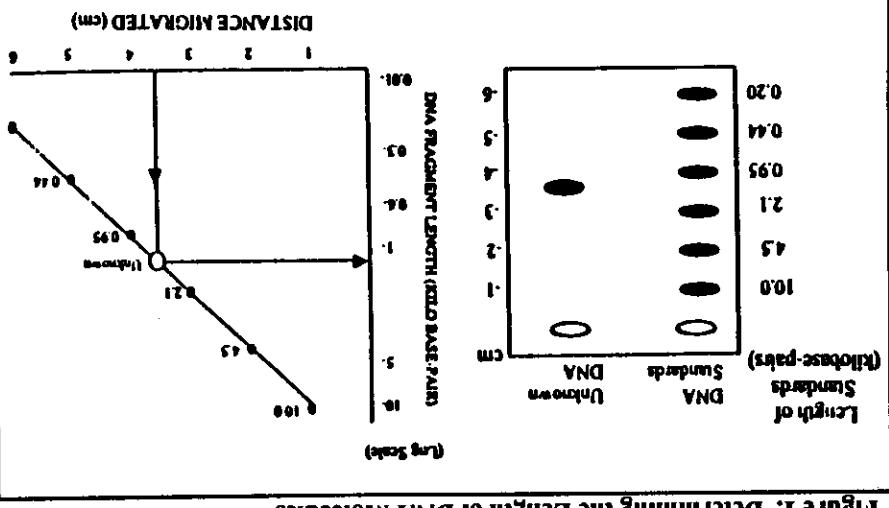


Figure 1. Determining the Length of DNA Molecules

Left: Six DNA standards and a DNA fragment of unknown length are electrophoresed on agarose lanes of a gel and the distances (in cm) that each has migrated during the run are determined. **Right:** Six distances measured from the gel are plotted against the logarithms of their lengths. The distances measured by the standards are plotted against the logarithms of their lengths. The length of the unknown is determined by extrapolation from the standard graph.

B. DETECTION OF LDH ACTIVITY IN AGAROSE GELS

Lactate dehydrogenase, like many other enzymes, is also found in serum where it is derived from normal cell death since dying and dead cells liberate their cellular enzymes into the bloodstream. The liberation of enzymes into the circulatory system is accelerated during tissue injury and the measurement of LDH isoenzymes in serum has been used extensively for determining the site and nature of tissue injury in humans. For example, when the blood supply to the heart muscle is severely reduced, as during a heart attack, muscle cells die and release LDH into the blood stream. Thus, an increase in LDH in serum is indicative of a heart attack.

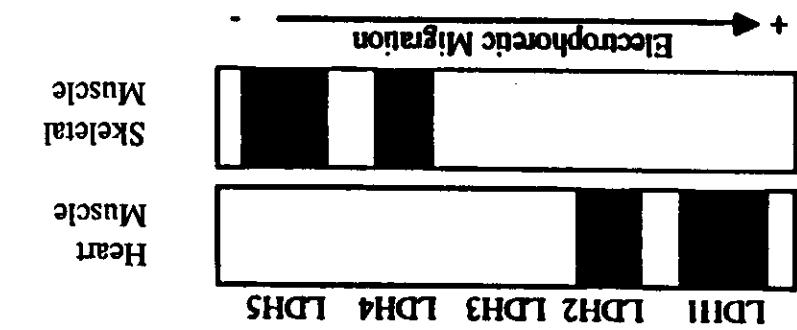


Figure 3-3. Electrophoretic Patterns of LDH Isoenzymes in Extracts from Heart and Skeletal Muscle.

- Place the staining tray in the refrigerator and allow 3-18 hours for staining.
- Decant and discard the stain, rinse the gel and dish with distilled water and add about 100ml of distilled water.
- Change the water after about 10-20 minutes and hold the staining dish over a light source such as a desk lamp or light box. Note the position of the dark blue DNA bands against the light blue gel background. The gel can be stored in water in the refrigerator for a few days. After about a week or two in the refrigerator, the dye will diffuse out of the gel and the DNA bands will no longer be visible.

Rapid Procedure

- Dilute the gel stain concentrate 1000-fold and stain the gels for 30-60 minutes in 100mls at 37°C.
- Decant and discard the stain, rinse the gel and dish with water and add about 100ml of distilled water.
- Change the water after about 20 minutes and again after an additional 20 minutes. DNA bands can been seen during these destaining steps.
- When the background stain has been reduced sufficiently, hold the staining dish over a light source and carefully observe the stained DNA bands in the gel.

Gel Storage

- The gel can be stored in a sealed plastic bag (3x3") with a few mls of distilled water for up to one month in the refrigerator.
- For long term storage, place the gel on a glass slide and smooth with a gloved index finger to eliminate air bubbles between the gel and the slide. Allow the gel to dry onto the slide at room temperature for 3-4 days. Cover the dry gel film and glass slide with saran wrap.

V. Suggested Reading and Reference for Part A

Lewin, B. *Genes V*. Oxford University Press, Oxford New York Tokyo, 1994.

Jones, P. *Gel Electrophoresis: Nucleic Acids*. Chichester, West Sussex, UK, New York: Wiley 1995.

The highly colored TNBT - Formazan product localizes in the electrophoretic zones of LDH activity and the amount of brown color formed is quantitatively related to the level of LDH isoenzyme present.

Objectives: In this experiment, you will first prepare tissue extracts from calf thymus. You will then characterize the LDH isoenzymes in the tissue extracts and in calf serum.

Materials Provided:

Calf Thymus (15 grams)

Calf Heart LDH - A tissue extract from calf heart enriched in LDH.

Calf Muscle LDH - An extract from skeletal muscle enriched in LDH.

*Extraction Buffer - The buffer contains NaCl, Tris (pH 8.0) and the detergent Nonidet P-40.

Electrophoresis Sample Buffer - The sample buffer contains glycerol and bromophenol blue.

Calf serum - The serum is diluted in electrophoresis sample buffer.

LDH-Substrate - Prepared fresh as described below.

*Prepared as described in the Instructor Guide.

Materials Not Provided

*Materials required for electrophoresis including electrophoresis buffer, agarose, and gel destain solution.

8 mortars and pestles

8 pair of scissors

8 ice baths

4 gel staining trays

A water bath maintained at 37°C

Small centrifuge (Either a clinical centrifuge or a microcentrifuge can be used.)

8 centrifuge tubes

Sixteen 1.5ml tubes

A balance

1. I. Preparation of the Agarose Gels.
While the gels are cooling, prepare the tissue extracts as described below.
 2. Place 0.5g of calf thymus into a precooled mortar and cut the tissue into small (~1cm³) sections with scissors.
 3. The class should be divided into eight groups for the preparation of the tissue extracts.
 4. Place 0.5g of calf thymus into a precooked mortar and cut the tissue into small extracts.
 5. With the power supply off, connect the cables from the cell to the power supply, red to red (positive) and black to black (negative).
 6. Push the rocker switches on the power supply to "on" and "170V". The voltage will now remain constant at 170 volts during the run.
 7. Unless otherwise indicated, electrophoresis until the bromophenol blue in the gel. At 170V, this takes approximately 50 minutes.
 8. At the termination of the electrophoretic separation, shut off the power supply, disconnect the cables and remove the gel casting trays containing the gels.
 9. The buffer should be emptied from the electrophoresis cell and stored under refrigeration in a separate container until the next electrophoretic run. The same buffer should be used for at least 3 electrophoretic separations. However, fresh buffer should be employed for the preparation of all agarose gels. The agarose gel and two groups will share one gel.

I. Preparation of the Agarose Gels.

1. Four 1.2% agarose gels should be prepared by the class as described on page 12 of this manual.

II. Preparation of the Thymus Extracts.

2. While the gels are cooling, prepare the tissue extracts as described below.

1. Place 0.5g of calf thymus into a precooked mortar and cut the tissue into small (~1cm³) sections with scissors.

2. Add 2ml of cold extraction buffer and grind the tissue sections with the pestle until a homogeneous suspension is formed. The mechanical action of the pestle and the chemical action of a detergent (NP-40) that is present in the extraction buffer should disrupt the plasma membrane leaving the nuclear envelope intact. Pour about 1ml of the homogeneous into a small centrifuge tube and centrifuge for 5 minutes to pellet the nuclei.

3. Pour the supernatant into a clean tube, label the tube thymus extract, and then place the tube in an ice bath.

1. Obtain one tube, label it T (for thymus) and add 1.5μl of electrophoresis sample buffer to the tube.

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- Pour the melted agarose directly from the test tube onto the casting deck and return the test tube to the hot (but not boiling) water bath. The small amount of melted agarose left in the test tube will be used for sample application (see below). Insert the comb into the casting tray slots and push down gently on the top of the comb until resistance is encountered. The teeth of the comb will come to rest in the melted agarose about 0.2mm above the surface of the glass plate.
- After the gel has cooled for at least 15 minutes, remove the tape strips and carefully lift the comb straight up and away from the casting tray. The gel is now ready for sample application. Gels can also be stored for up to one week before use. For gel storage, the comb is left in place and the tray containing the gel and comb is wrapped in plastic wrap and placed in the refrigerator.

Sample Application

Prior to sample application, place the following items on the laboratory bench in front of you.

- *Agarose gel
 - *Sample for electrophoresis
 - *Micropipetor and micropipets
 - *Absorbent tissue (e.g. Kleenex or Kimwipes)
 - *Small beaker of distilled or deionized water
 - *Melted agarose - Transfer the melted agarose from the large test tube in the hot water bath to a small tube. The small tube should then be placed in a beaker of hot water to ensure that the agarose remains in a liquid state.
- Hold the micropipetor in a vertical position and place the filling end of the micropipet into the sample solution.
 - Draw the sample into the pipet to the 15 μ l calibration line by lifting up on the handle of the plunger assembly.
 - Wipe excess liquid from the outer pipet surface with an absorbent tissue.
 - Carefully direct the filling end of the micropipet into the top of the sample well and slowly eject the 15 μ l of the sample well.

5. Draw melted agarose into the micropipet to the 20 μ l calibration line, direct the filling end into the sample well, and slowly eject the agarose onto the sample until the well is full. Between 10-20 μ l of agarose are required to fill the well. The agarose will seal the sample in the sample well.

- Rinse the pipet by drawing up and expelling water three times from the pipetor.
- Wipe excess liquid from the outer pipet surface with an absorbent tissue.
- Repeat steps 1-7 to load each additional sample.

- Load 15 μ l of the following samples into the agarose gel sample wells.

Sample Well Number	Sample	
1	Calf Serum	Group 1
2	Calf Heart LDH	
3	Calf Muscle LDH	
4	Calf Thymus Extract	
5	Calf Serum	Group 2
6	Calf Heart LDH	
7	Calf Muscle LDH	
8	Calf Thymus Extract	

- Electrophoresis at 170V until the bromophenol blue in the tissue extract samples has migrated to within 1mm of the positive electrode end of the gel.
- Remove the gels from the electrophoresis cell, rinse them in distilled water, and note the blue serum albumin bands in lanes 1 and 5. Some of the bromophenol blue in the sample will remain bound to albumin during the electrophoretic run.

IV. Detection of LDH Isoenzymes

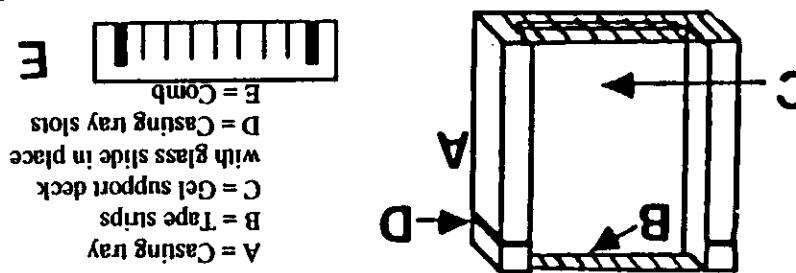
- Preparing the LDH Substrate Solution: The instructor should prepare the LDH substrate solution at the end of the electrophoretic run. About 0.5ml of packed substrate is provided which can be used for two separate experiments. To prepare the substrate solution for this experiment, remove about half of the substrate powder from the tube with a spatula and dissolve the powder in 65ml of distilled water. Return the remaining powder to the freezer.
- Add 15ml of the LDH-substrate solution to a gel staining dish containing your gel and incubate in the dark for 30-40 minutes at 37°C. Do not mouth pipet this solution.

Pouring the Agarose Gels

The exercises described in this manual were designed such that the samples of two students are analyzed on one agarose gel. If students work in pairs, four students will share one gel. Four agarose gels are electrophoresed simultaneously using the Procell and MB-170 power supply.

IV. PROCEDURES FOR THE PREPARATION, ELECTROPHORESIS AND STAINING OF AGAROSE GELS

- *The melted agarose for the four gels (15ml per gel) can also be prepared in one bunser burner or in a microwave oven. The flask should be rotated periodically during the heating process in order to prevent damage to the agarose.
- *The agarose solution by boiling 0.9g of agarose in 75ml of buffer in a 250-500ml flask over a flame to come to a vigorous boil. After boiling for about two minutes, remove the test tube from the bath, stir gently with a glass rod, and cool at room temperature for about 2-3 minutes. At this time, the agarose solution should be absolutely clear.
- 4. Place the test tube into a boiling water bath and allow the agarose suspension to come to a vigorous boil. After boiling for about two minutes, remove the test tube from the bath, stir gently with a glass rod, and cool at room temperature for about 2-3 minutes. At this time, the agarose solution should be absolutely clear.
- 3.* With the macro pipetor (piper-syringe), dispense 15ml of electrophoresis buffer into a 25ml glass test tube and add 0.18 grams of agarose. The agarose can be weighed out directly on an appropriate balance. If a balance is not available, weigh the agarose until two-thirds full. Gently swirl the glass tube until the agarose forms a suspension.
- 2. Place the test tube into a boiling water bath and add 0.18 grams of agarose. Remove the test tube from the bath, stir gently with a glass rod, and cool at room temperature for about 2-3 minutes. At this time, the agarose solution should be absolutely clear.



CASTING TRAY ASSEMBLY

1. Place the casting tray on a level work surface and place a precleaned glass slide into the gel support deck.
2. Seal both ends of the gel support deck with tape. The tape must be firmly pressed against the edges of the deck to ensure a tight seal.

Estimates of the % of Total LDH Activity*

- | SAMPLE | LDH-1 | LDH-2 | LDH-3 | LDH-4 | LDH-5 |
|--|-------|-------|-------|-------|-------|
| Serum | | | | | |
| Thymus | | | | | |
| Heart | | | | | |
| Muscle | | | | | |
| Skeletal Muscle | | | | | |
| 3. The gels can be stored in standard desalting solution containing 10% methanol and 5% acetic acid. | | | | | |
| *These percentages can be determined accurately with a gel densitometer. | | | | | |
| 1. In which sample(s) do you find the highest amount of the polypeptide chain H? | | | | | |
| 2. Explain why an elevation in the level of a serum enzyme may be indicative of a disease state. | | | | | |
| 3. What role does the Nondicot P-40 serve in the extraction buffer? | | | | | |

Data Analysis

IV. PROCEDURES FOR THE PREPARATION, ELECTROPHORESIS AND STAINING OF AGAROSE GELS

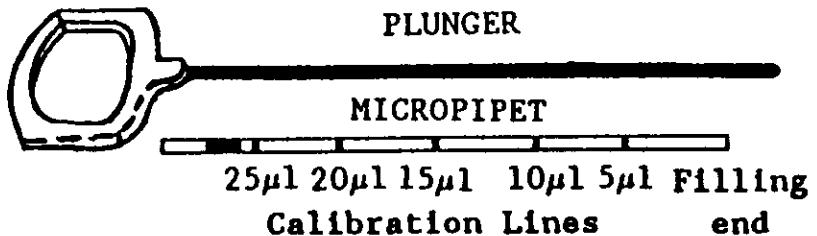
- *The agarose solution by boiling 0.9g of agarose in 75ml of buffer in a 250-500ml flask over a flame to come to a vigorous boil. After boiling for about two minutes, remove the test tube from the bath, stir gently with a glass rod, and cool at room temperature for about 2-3 minutes. At this time, the agarose solution should be absolutely clear.
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- 3.* With the agarose (15ml per gel) can also be prepared in one bunser burner or in a microwave oven. The flask should be rotated periodically during the heating process in order to prevent damage to the agarose.
- 2. Rinse the gels with water and examine them on a light box. Locate the bands containing LDH-1, LDH-2, LDH-3, LDH-4, and LDH-5. Figure 3-3 should help in this identification.
- 1. The amount of brown color is roughly proportional to the amount of an LDH isoenzyme present in a band. In the table below, estimate the amounts of each isoenzyme present in each protein sample.

IV. PROCEDURES FOR THE PREPARATION, ELECTROPHORESIS AND STAINING OF AGAROSE GELS

- *The agarose solution by boiling 0.9g of agarose in 75ml of buffer in a 250-500ml flask over a flame to come to a vigorous boil. After boiling for about two minutes, remove the test tube from the bath, stir gently with a glass rod, and cool at room temperature for about 2-3 minutes. At this time, the agarose solution should be absolutely clear.
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- 1. The amount of brown color is roughly proportional to the amount of an LDH isoenzyme present in a band. In the table below, estimate the amounts of each isoenzyme present in each protein sample.

The micropipets are an important accessory used for electrophoresis procedures. The micropipet consists of a stainless steel plunger and 50 micropipets with calibration lines of 5 μ l*, 10 μ l, 15 μ l, 20 μ l, and 25 μ l as shown below.

A Diagram of the Micropipet Apparatus



*One milliliter (ml) = 1,000 microliters (μ l)

TO OPERATE THE MICROPIPETOR:

1. Insert the metal plunger into the end of the glass pipet that is opposite the calibration lines. The glass pipet can be held between your thumb and middle finger and the plunger operated with your index finger on the same hand.
2. Gently push down on the plunger until the plunger handle comes to rest on the pipet.
3. Hold the micropipet in a vertical position and place the filling end into the sample solution.
4. Draw the sample into the pipet to the appropriate calibration line by lifting up on the handle of the plunger assembly.
5. Carefully wipe excess liquid from the outer pipet surface with an absorbent tissue.
6. Direct the filling end of the micropipet into a tube and slowly eject the sample.
7. Rinse the pipet between samples by drawing up and expelling water three times from the pipetor.

Students should practice using these pipets prior to beginning the experiments.

Experiment 1 (701). Enzyme Cytochemistry

General Background

A. ENZYME CYTOCHEMISTRY

Most cells are colorless, translucent, and almost invisible in an ordinary light microscope. Consequently, biologists frequently stain cells to render them visible for microscopy. A number of organic dyes are available which can be used to stain biological specimens and some of these stains show a preference for particular parts of the cell. For example, the basic dyes methylene blue and toluidine blue stain cell nuclei, acid dyes like eosin stain the cytoplasm, suds black stains fat droplets and janus green stains mitochondria. Although these dyes are useful for identifying cell organelles, they lack the specificity required to detect individual macromolecules.

A variety of new methods are now used by cell biologists for detecting specific macromolecules at the light microscopic level and you will use one of these methods in today's laboratory. The method is called enzyme cytochemistry, which permits the localization of specific enzymes in cells or tissues. Enzyme cytochemistry can be used to study the subcellular distribution of specific enzymes and can tell us if an enzyme is found in a particular organelle. In addition, the method is a powerful tool for identifying specific cell and tissue types.

The basic steps in enzyme cytochemistry are diagrammed in Figure 1-1 and described below.

1. Fixation

Cells or tissues are usually "fixed" by immersion in acids, organic solvents such as ethanol or acetone, or reactive aldehydes. Cells are fixed prior to most staining procedures in order to make them permeable to the stains. In addition, fixation causes cells to adhere to the microscope slide and cross-links cell proteins which locks or "fixes" them in position.

2. Incubation with a special colorless enzyme substrate

Fixed cells or tissue sections are incubated with a colorless substrate of the enzyme under study and the enzyme catalyzes the conversion of the substrate to a colored product.

3. Detection of the colored product by microscopy

The colored product deposited at the site of enzyme action is then detected by microscopy.

Figure 1-1. Enzyme Cytochemistry.

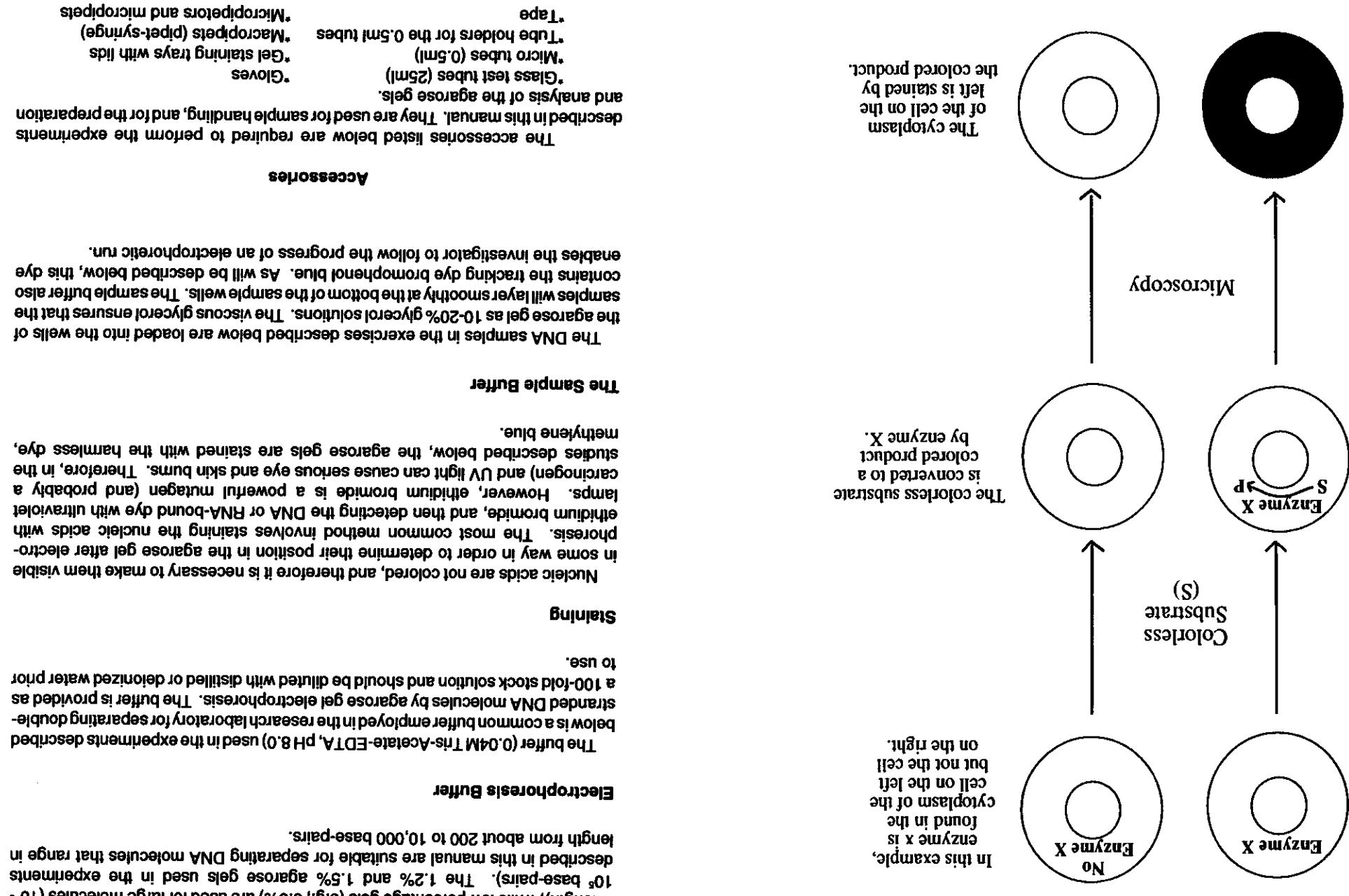
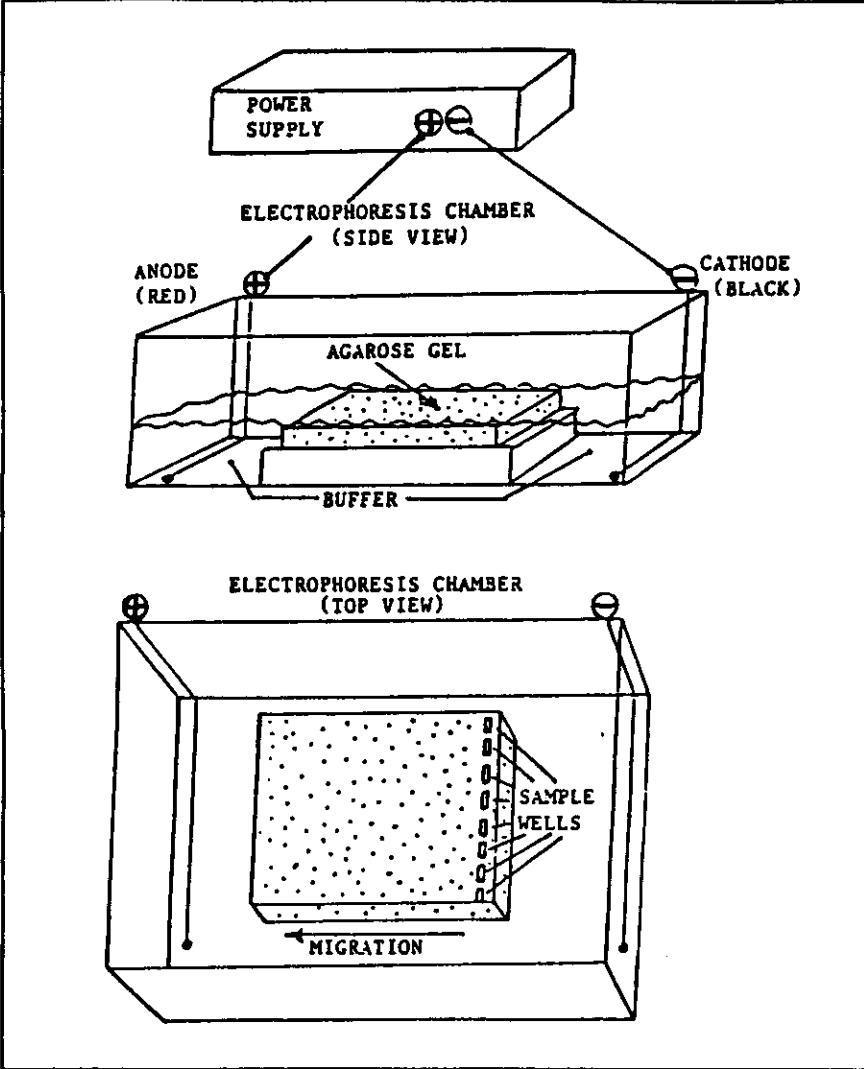


Figure 7. Components of a Horizontal Electrophoresis System.



Electrophoresis Chemicals

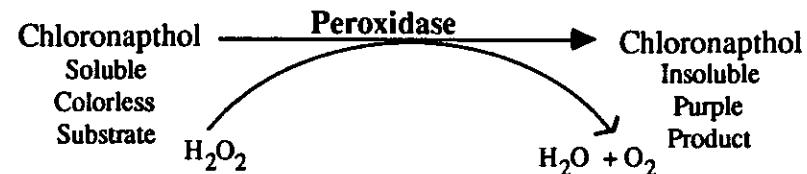
The Agarose Gel

Because the agarose gel is an ideal solid support for the separation of nucleic acids on the basis of size, it is used extensively for this purpose in the molecular biology laboratory. Agarose is a natural polysaccharide of galactose and 3,6-anhydrogalactose derived from agar, which, in turn, is obtained from certain marine red algae. Agarose gels are made by dissolving the dry polymer in boiling buffer, pouring the gels into casting trays and allowing them to set by cooling at room temperature. The resolving power of an agarose gel depends on the pore size, which

B. PEROXIDASE

The enzyme that you will study in Parts A and B of today's laboratory is called peroxidase. Peroxidase catalyzes the oxidation of phenolic compounds at the expense of hydrogen peroxide (H_2O_2). Although hundreds of papers have been published on peroxidase, the precise functions of the enzyme are uncertain. In plant systems, peroxidase is likely to play a role in synthesis of the plant cell wall. Here, the enzyme cross links phenolic residues of cell wall polysaccharides and glycoproteins which serves to strengthen the cell wall components. Peroxidase can also kill microorganisms and destroy chemicals that are toxic to both plant and animal cells including H_2O_2 , phenols, and alcohol. For these reasons, it has been proposed that peroxidase protects cells from microorganisms and toxic chemicals.

In this laboratory, you will localize peroxidase in cells and tissues by enzyme cytochemistry. The reaction that will be exploited is shown below. Cells or tissues will be incubated with chloronaphthol and hydrogen peroxide. The peroxidase converts the chloronaphthol to an insoluble purple product which is deposited at the site of the enzyme.



Objectives In this three-part exercise, you will localize peroxidase at the cell and tissue level by enzyme cytochemistry. In Part A, peroxidase will be localized at the tissue level in germinating corn seeds. In Part B, the intracellular location of the enzyme in onion epithelial cells will be examined. The cell types containing peroxidase in blood will then be identified in Part C.

Laboratory Schedule

Sufficient materials are provided for 8 groups of students to perform the experiments. Each part of this exercise requires between 30-40 minutes to complete and the different parts can be performed in different laboratory sessions.

The Model MB-170 power supply is a general purpose electrophoresis power source. The unit produces a constant voltage output of 85 or 170 volts. Voltage selection is controlled by the switch located in the center of the front panel. The ammeter, also located on the front panel, permits the current to be monitored during an electrophoretic run. The unit can reach a maximum of 500 mAmp.

The PROCCELL Horizontal Electrophoresis unit is composed of an acrylic cell with a central platform, platinum electrodes, four removable gel-casting trays, four sample wells-forming combs and a safety lid with power cords. The four gels are made in the casting trays and then placed on the central platform of the electrophoresis cell. Each gel contains 8 separate sample wells. The experiments described below were designed such that each student uses four sample wells per experiment. Therefore, the experiments of 8 students can be analyzed in one electrophoretic run. If the students work in pairs, the system can be used by 16 students.

Electrophoresis Equipment

III. Practical Aspects of Electrophoresis

At neutral pH, a molecule of DNA or RNA is negatively charged because of the negative charges on the phosphate backbone. Under these conditions, nucleic acids applied to sample wells at the negative electrode end of the gel migrate within pores of the gel matrix towards the positive electrode. The agarose gel serves as supports for the gel matrix towards the positive electrode. The agarose gel matrix has pores smaller than the size of the DNA molecules. Thus, the method sorts the molecules slowly through the pores of the gel according to size.

Electrophoresis is the movement of charged particles in solution under the influence of an electric field. In the most common form of electrophoresis, the sample molecules travel through a gel matrix. The gel is a common type of stabilizing medium used for the electrophoretic separation of nucleic acids. A diagram of the essential components of an agarose electrophoretic system is shown in Figure 7. The agarose gel, containing sample wells, is submerged in buffer within the electro-
chamber. Samples to be separated are then loaded into the sample wells.
Current from the power supply travels to the negative electrode (cathode), supplying electrons to the conductive buffer solution, gel and positive electrode (anode), thus completing the circuit.

II. General Description of Agarose Gel Electrophoresis

Mature seeds have a low water content and the cells of the embryo are biochemically dormant. The cells can be activated in seeds by environmental factors, especially in increase in the moisture content of the atmosphere. This activation is called germination which begins by the uptake of water (imbibition phase) and culminates in the pronusion of the embryonic root from the seed. In corn, germination requires 24-48 hours under ideal conditions, and can be divided into the

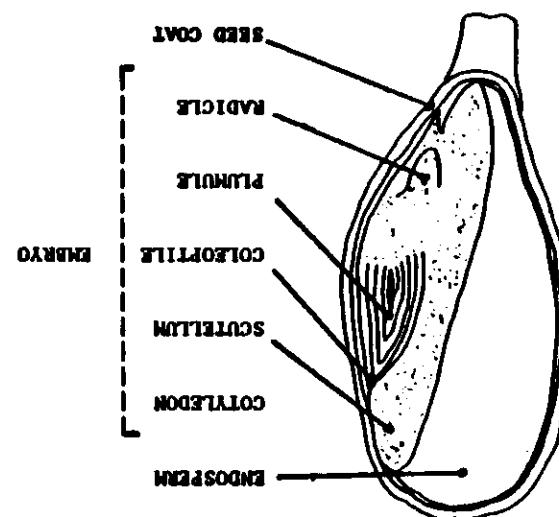


Figure 1-2. Longitudinal Section of the Corn Seed.

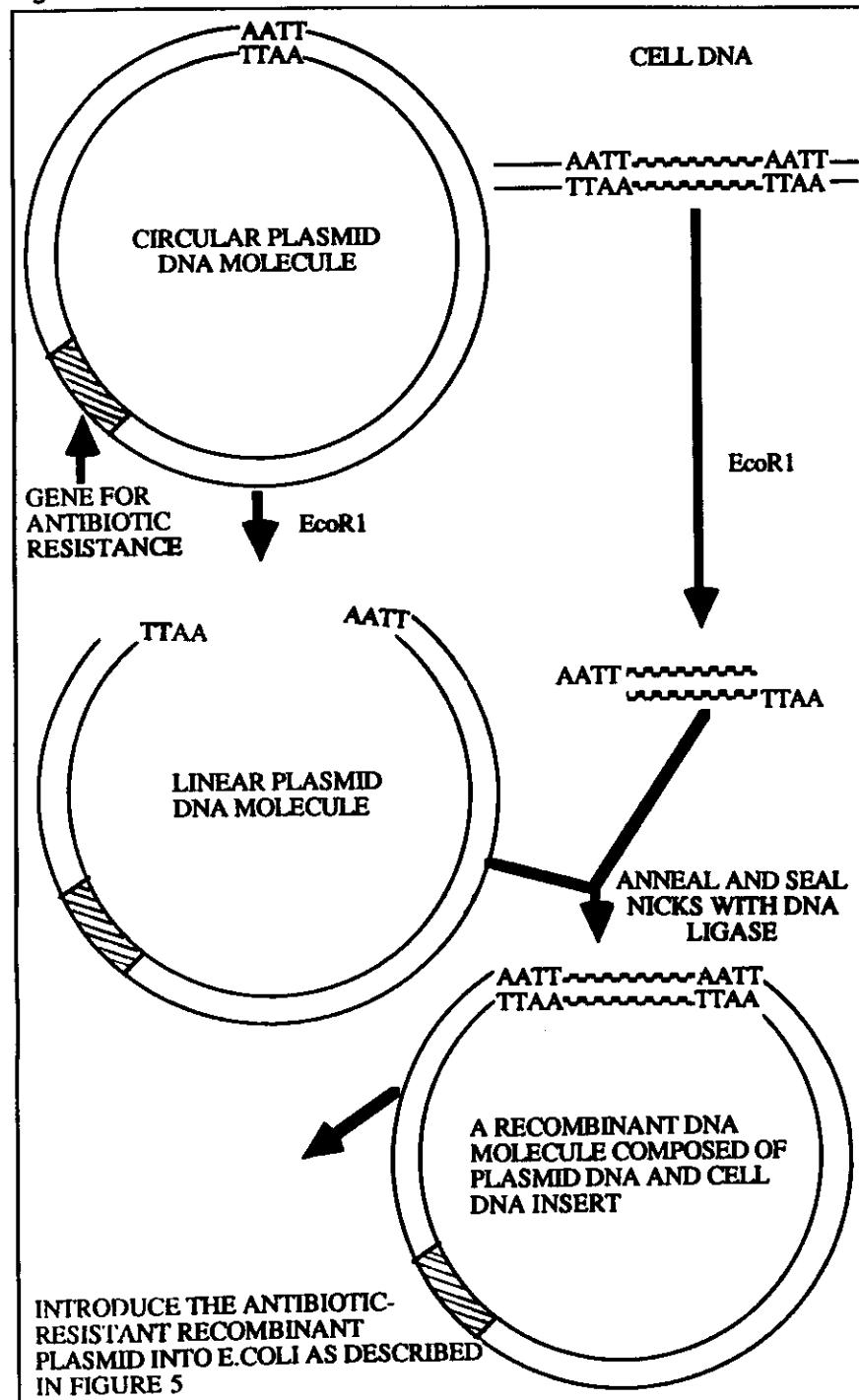
The seeds of flowering plants are typically resistant structures in which embryo plants are enclosed. The outer layer of the seed is called the seed coat which protects the embryo from adverse conditions. The structures of the seed coat of embryo plants are similar in that each seed contains a seed coat, an embryo, and a food storage tissue. The structures in a mature com seed are shown in Figure 1-2. The mature com embryo has a single cotyledon, or seed leaf, which is made up of a food-absorbing portion called the scutellum and the cotyledone which forms a protective cap over the shoot. Below the cotyledon is the plumule, the apex of the embryonic shoot. The basal end of embryo, the radicle, develops into the primary root when the seed germinates. The food for the development of the com seedling is largely located in the endosperm. In fact, the embryo is embedded in the cellular endosperm, the cells of which are rich in stored protein and especially starch. The endosperm is surrounded by several layers of cells that form the aleurone layer, in which a variety of enzymes are produced during germination.

A. SEED GERMINATION

Objective - Part A: To identify the sites of respiration, starch storage, and peroxidase in the germinating com seed.

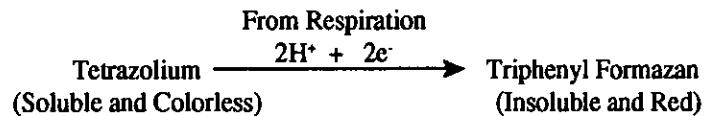
Part A. Peroxidase in the Germinating Corn Seed

Figure 6. Production of a Recombinant DNA Molecule



1. Imbibition of water and hydration of subcellular organelles
2. Activation and new synthesis of a variety of enzymes in the aleurone layer and in the embryo
3. An increase in the levels of digestive enzymes amylase and proteinases in the aleurone layer and scutellum. These enzymes break down the stored starch and proteins into glucose and amino acids in the endosperm.
4. The transport of the breakdown products (glucose and amino acids) from the endosperm to the embryo where they are used as a nutrient source for the growing embryo
5. An increase in oxygen uptake and respiratory activity of the embryonic cells
6. Cell division and differentiation in the embryo and the emergence of the root from the seed.

Seed-testing laboratories frequently determine the percentage of viable (living) seeds in a seed lot by growing seeds under prescribed conditions and observing the number that germinate. The activation of respiration during germination forms the basis of a simple alternative test for seed viability. In this test, seeds are incubated with a colorless tetrazolium dye. Electrons produced by cellular respiration reduce the dye to a red product as shown in the equation below. Tetrazolium reduction has been used to localize respiring tissues in plants and animals and to detect mitochondria in viable cell preparations. In this exercise, you will use this histochemical procedure to identify the sites of respiration in the germinating corn seed.



Objective - Part A To identify the sites of respiration, starch storage, and peroxidase in the germinating corn seed.

INTRODUCE THE ANTIBIOTIC-RESISTANT RECOMBINANT PLASMID INTO E. COLI AS DESCRIBED IN FIGURE 5

Typically, the restriction sites for a given enzyme are hundreds to thousands of base-pairs apart so that the fragments generated are hundreds to thousands of base-pairs long. More than 300 different restriction nucleases are now commercially available. General properties of three of these enzymes are given in Table 1. It should be noted that some restriction nucleases (e.g., EcoRI and BamHI) produce staggered cleavage that creates sticky, or cohesive, single-stranded ends on the cut molecules. These cohesive ends are very important in recombinant DNA procedures because they enable any two DNA fragments to be linked together by complementary base pairing at their ends, provided that they were generated with the same restriction enzyme.

Figure 6 illustrates one basic procedure by which cellular DNA from essential

DNA fragments can then be recovered by cleavage of the recombinant plasmid with EcoRI. Digestion of DNA from a vertebrate cell with EcoRI generates about 10^6 different DNA fragments. Thus, the DNA cloning procedure described above and outlined in Figure 6 gives rise to a large number of plasmids, each descended from a single hybrid DNA molecule. The most difficult step in the procedure is to identify the hybrid plasmid in this library that contains the inserted cellular DNA of interest. When a particular mRNA can be purified from a tissue, such as the MRNAs for the polypeptide chains of hemoglobin, the mRNA or a DNA copy of it can be used to identify its corresponding gene sequence in a recombinant library. In this procedure, the DNA clone of interest because of complementary base-pairing. Under the procedure, the genes for many different proteins in DNA libraries have been identified.

- Preparation of Germinalizing Com Seeds**

Note: This procedure must be performed 1-2 days before the laboratory session. The procedure can be performed by the instructor, in which case about 50 seeds should be prepared, or by 8 groups of students who should prepare about 5-6 seeds each.

1. Place a layer of filter paper or paper towels in a petri dish and add about 5 ml of water to moisten the paper.

2. Add about 5-6 seeds and cover the dish. The seeds will be ready for analysis after 1-2 days in the dish.
- Automy of Germinalizing Com Seeds**

Using a razor blade, cut a germinating corn seed in half, lengthwise from one flat side to the other. Note the seed coat, the position of the aleurone layer between the endosperm and the seed coat, the endosperm, and the small triangular section of the embryo with a hand lens or binocular microscope and section of the embryo with a hand lens or binocular microscope and attempt to identify the cotyledon, plumule, and radicle. (See Figure 1-2)

*Prepared as described in the instructor manual.

Materials Provided

- Germinalizing Com Seeds (Note: the seeds must be soaked as described below 1-2 days before the laboratory session.)
- *Tetrazolium (Freshly prepared)
- *Potassium iodide
- *Peroxidase Substrate Solution (Freshly prepared) - This solution containing hydrogen peroxide, chloromaphol and Tris buffer should be made up 1-10 minutes before the experiment.
- *Peroxidase Substrate Solution (Optional) - This solution containing hydrogen peroxide, chloromaphol and Tris buffer should be made up 1-10 minutes before the experiment.
- Razor blades or scalpels
- Hand lenses or binocular low power microscopes (optional)
- Distilled or deionized water
- A water incubator maintained at 37°C (optional)
- 32 small petri dishes (e.g. 3.5 x 1.0cm) or 32 small tubes (e.g. 1.5ml micro tubes).

Materials Not Provided

- Transfer pipets
- Procedure

Typically, the restriction sites for a given enzyme are hundreds to thousands of base-pairs apart so that the fragments generated are hundreds to thousands of base-pairs long. More than 300 different restriction nucleases are now commercially available. General properties of three of these enzymes are given in Table 1. It should be noted that some restriction nucleases (e.g., EcoRI and BamHI) produce staggered cleavage that creates sticky, or cohesive, single-stranded ends on the cut molecules. These cohesive ends are very important in recombinant DNA procedures because they enable any two DNA fragments to be linked together by complementary base pairing at their ends, provided that they were generated with the same restriction enzyme.

- *Prepared as described in the instructor manual.
- Materials Provided**
- Germinalizing Com Seeds (Note: the seeds must be soaked as described below 1-2 days before the laboratory session.)
 - *Tetrazolium (Freshly prepared)
 - *Potassium iodide
 - *Peroxidase Substrate Solution (Freshly prepared) - This solution containing hydrogen peroxide, chloromaphol and Tris buffer should be made up 1-10 minutes before the experiment.
 - Peroxidase Substrate Solution (Optional) - This solution containing hydrogen peroxide, chloromaphol and Tris buffer should be made up 1-10 minutes before the experiment.

A nuclease is an enzyme that breaks the phosphodiester bonds that connect the nucleotide units in DNA or RNA. Restriction nucleases are powerful tools used in recombinant DNA technology because they cut DNA at specific sites. These enzymes are produced chiefly by bacterial species in which they serve to degrade invading foreign DNA within the bacterial cell. Most restriction enzymes recognize a specific sequence of four to six nucleotides in DNA and each will cut a long DNA double helix into a series of discrete pieces known as restriction fragments.

Table 1. Properties of Three Restriction Enzymes

Restriction Nuclease	Bacterial Source	Recognition Sequence and Mode of Cleavage
Hpa I	<i>Haemophilus parainfluenzae</i>	<p>- G - T - T - A - A - C - - C - A - A - T - T - G -</p> <p>Cuts on lines of symmetry</p> <p>- G - T - T A - A - C - - C - A - A T - T - G -</p> <p>Blunt ends</p>
EcoR1	<i>Escherichia coli</i>	<p>- G - A - A - T - T - C - - C - T - T - A - A - G -</p> <p>Staggered Cleavage</p> <p>- G A - A - T - T - C - - C - T - T - A - A G</p> <p>Cohesive (Sticky) Ends</p>
Bam H1	<i>Bacillus amyloliquefaciens</i>	<p>- G - G - A - T - C - C - - C - C - T - A - G - G -</p> <p>Staggered Cleavage</p> <p>- G G - A - T - C - C - - C - C - T - A - G G</p> <p>Cohesive (Sticky) Ends</p>

III. Location of starch, peroxidase, and the sites of respiration in seeds.

- Obtain four small tubes or dishes and label them #1 - #4 with a marking pen.
- Using a large transfer pipet, place 1ml of the following into the tubes. (Do not mouth pipet these solutions.) The iodine is a stain for starch, tetrazolium for respiration activity, and the peroxidase substrate for peroxidase.

Tube Number	
#1	Water
#2	Iodine
#3	Tetrazolium Solution
#4	Peroxidase Substrate Solution

- Using a razor blade, cut 4 seeds in half as described above, and rinse the seed halves in water.
- Place two halves in the solutions in each tube or dish such that the cut surfaces are in contact with the solutions. The seeds should be transferred with forceps or by piercing them with toothpicks.
- After 3 minutes, remove the seeds from tube #2, rinse the seed halves in water, and note the seed areas that have stained with iodine and those that have not. Note - use forceps or toothpicks to transfer the seeds.
- After 20-30 minutes, use forceps or toothpicks to remove the seeds from tube #1, 3, and 4. This time period can be reduced to 10 minutes if the incubation is carried out at 37°C. Rinse the seeds in water and note the colored seed areas in treatment groups 3 and 4. A hand lens or a binocular microscope can be used to study the colored areas of all seeds in more detail.

Data Analysis

In the space provided below, record the seed areas that are stained by each treatment.

Tube Number	Treatment	Result
1	H_2O	
2	Iodine	
3	Tetrazolium	
4	Peroxidase Substrate	

1. A bulb is a short stem wrapped in fleshy modified leaves which are called bulb scales. The scales are covered with a single layer of epidermal cells which will

Procedure

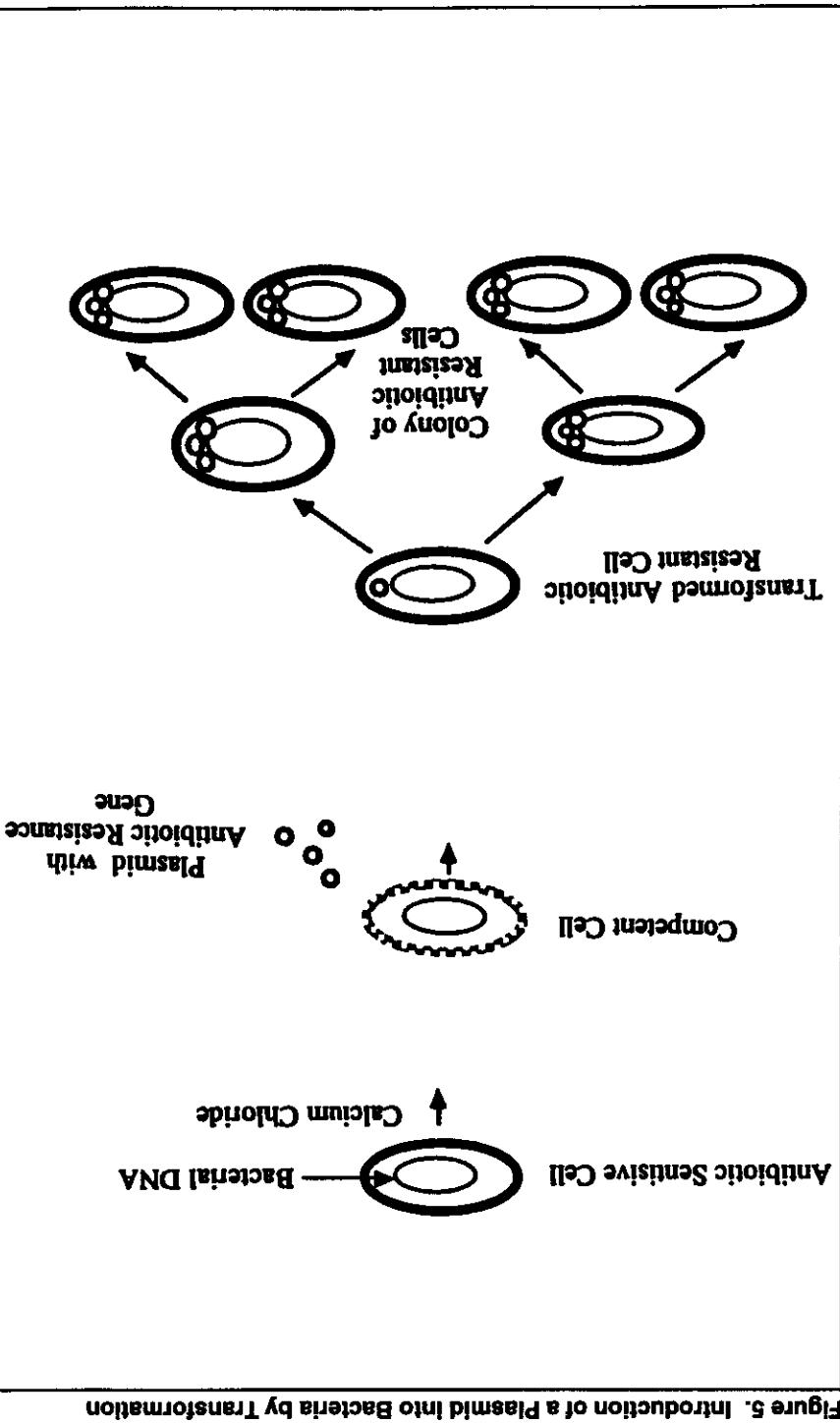


Figure 5. Introduction of a Plasmid into Bacteria by Transformation

Part B. Intracellular Localization of Peroxidase

Two approaches are frequently used to locate a specific enzyme within a cell. The first involves isolating cell fractions that contain different organelles by physical techniques such as centrifugation. The amount of the protein in each fraction is determined by a biochemical procedure (e.g. an enzyme assay). For example, a mitochondrial fraction can be prepared from cells by differential centrifugation and the mitochondrial enzyme should be found only in this cell fraction. The second approach consists of the direct detection of the enzyme in the cell by a specific staining procedure like enzymatic staining. Only in this cell fraction, the specific site of the enzyme should be found. For example, a mitochondrial fraction can be prepared from plant cells by differential centrifugation and the specific site of the enzyme should be found only in the mitochondria. Both approaches have been used to localize peroxidase to a specific site in plant cells, and you will now definitely determine the subcellular site of an enzyme. Both approaches have been used to localize peroxidase to a specific site in onion epidermal cells by enzymatic staining. The second approach consists of the direct detection of the enzyme in the cell by a specific staining procedure like enzymatic staining. Only in this cell fraction, the specific site of the enzyme should be found only in the mitochondria. Both approaches have been used to localize peroxidase to a specific site in onion epidermal cells by enzymatic staining.

Objective-Part A To localize the site of peroxidase in onion epidermal cells by enzymatic staining.

Onion Bulb - The bulb should be cut into about 10 sections at the beginning of the experiment.

*Peroxidase (from Part A)

*Hydrogen Peroxide Substrate Solution (Freshly prepared) - This solution containing minutes before the experiment.

*Transfer Pipes

*Prepared as described in the instructor manual

Materials Not Provided

Razor blades or scalpels

Distilled or deionized water

Microscopes

Forceps

Cover slips

Dissecting probes, straight pins, or needles

Ethyl alcohol

Microscope slides (Slides with frosted ends are preferred.)

Forceps

Microscopes

Forceps

Distilled or deionized water

Microscopes

Forceps

Cover slips

Dissecting probes, straight pins, or needles

Ethyl alcohol

Analysis of Specific Genes and Recombinant DNA Technology

A key to one of life's great mysteries was discovered in 1953 when the double-helical structure of DNA was perceived by Watson and Crick. Elucidation of the basic mechanisms of replication, transcription and translation quickly followed, and by the early 1960's, the model shown in Figure 4 was generally accepted by most biologists. However, genes from higher organisms resisted detailed analysis until the mid 1970's because of the complexity of the DNA in eukaryotic organisms; a vertebrate cell contains enough DNA to code for more than 100,000 proteins. In order to study the structure and function of a single protein coding gene, the gene must be prepared in a purified form. The isolation of a specific gene from cellular DNA by conventional biochemical procedures is not practical because of the magnitude of the purification required (usually 100,000-fold) and because the procedures would necessitate the use of a large quantity of starting cellular DNA. Herein lies the major use of recombinant DNA technology, for it permits the amplification and isolation of specific genes by relatively simple procedures. A basic understanding of these procedures requires a description of an interesting feature of bacterial physiology.

Plasmids are small circular DNA molecules that exist apart from the chromosomes in most bacterial species. Under normal circumstances, plasmids are not essential for survival of the host bacteria. However, many plasmids contain genes that enable the bacteria to survive and prosper in certain environments. For example, some plasmids carry one or more genes that confer resistance to antibiotics. A bacterial cell containing such a plasmid can live and multiply in the presence of the drug. Indeed, antibiotic-resistant *E. coli* isolated in many parts of the world contain plasmids that carry the genetic information for protein products that interfere with the action of many different antibiotics.

In the laboratory, plasmids can be introduced into living bacterial cells by a process known as transformation. When bacteria are placed in a solution of calcium chloride, they acquire the ability to take in plasmid DNA molecules. As illustrated in Figure 5, this procedure provides a means for preparing large amounts of specific plasmid DNA since one transformed cell gives rise to a clone of cells that contains exact replicas of the parent plasmid DNA molecule. Following growth of the bacteria in the presence of the antibiotic, the plasmid DNA can readily be isolated from the bacterial culture.

Plasmids, as well as certain viruses, are extraordinarily useful tools for the molecular biologist, because they serve as gene-carrier molecules called cloning vectors. A basic procedure of recombinant DNA technology consists of joining a gene of interest to vector DNA to form a hybrid or recombinant molecule that is able to replicate in bacteria. Thus, cloning vectors contain genes for replication in bacteria. In addition, vectors generally carry antibiotic-resistance genes so that uninfected bacteria can be eliminated from the culture. In order to prepare a recombinant DNA molecule, a procedure is required for cutting cloning vectors and cellular DNA molecules at precise positions.

be used for the experiment. Remove one of the fleshy "scale" leaves from an onion bulb section and place it on the bench in front of you, concave surface up.

2. Obtain three clean glass slides and label them #1-#3 with a pencil.
3. With forceps, peel off three small pieces of the outer epidermal layer (~ 1cm x 2cm) and place one piece evenly onto each slide. If the tissue becomes badly wrinkled, discard it and obtain a new piece.
4. Place a few drops of ethyl alcohol onto the epidermis on each slide in order to fix the tissue. Add fresh alcohol to the tissue after 1 minute, and then let the slides air dry for a few minutes.
5. Place a few drops of the iodine solution onto the tissue on slide #1, water on slide #2, and Peroxidase Substrate on slide #3. Iodine staining is done to highlight internal structures of the cells.
6. After about 8 minutes, add a few additional drops of iodine onto slide #1, water onto slide #2, and Peroxidase Substrate onto slide #3.
7. After 10 minutes, place a paper towel at the edge of the tissue sections to draw off the solutions on the slides. Place a few drops of water onto each tissue section and draw off the water using a paper towel. Note the color of the tissue sections.
8. Place a few drops of water onto each section. Using a dissection probe or needle, slowly lower a cover slip over the tissue sections attempting to avoid trapping air bubbles.
9. Examine slide #1 with the low-power objective and then examine the cells under high power. Identify the cell walls that surround the individual cells, the cytoplasm, and finally the nuclei which should appear as brown staining bodies in the translucent cytoplasm. Next, examine slides #2 and #3, identify the above structures, and carefully note the subcellular distribution of the purple color in slide #3.

Data Analysis

The student should provide a written description or a drawing of the subcellular distribution of peroxidase activity (purple color) on slide #3.

The mRNA associates with ribosomes and its nucleotides are matched three at a time to a complementary set of three nucleotides in a specific tRNA molecule. The tRNA molecule carries an amino acid and when it associates with the mRNA, the amino acid is added to the growing polypeptide chain.

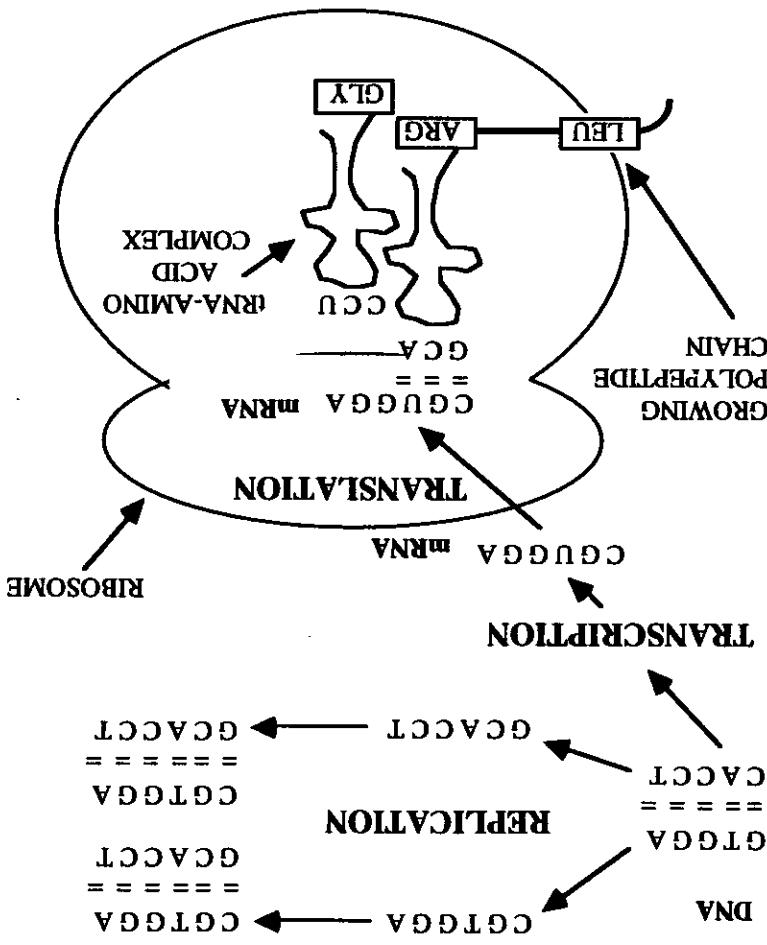


Figure 4. Molecular Information Transfer: Complementary Base Pairing

$$\text{NAD}^+ + \text{Nicotinamide-adenine dinucleotide} \rightarrow \text{NADH} + \text{H}^+ + 2 \text{ electrons} + 2 \text{ protons}$$

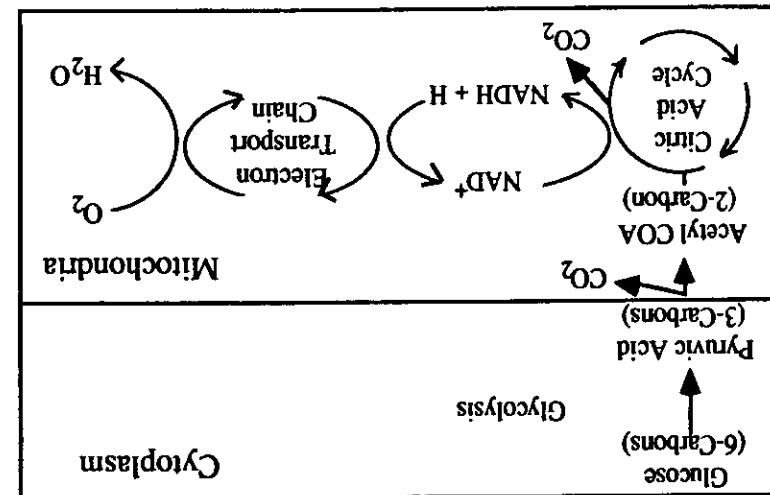


Figure 1-3. Summary of Respiratory Metabolism.

A. METABOLISM AND LACTATE DEHYDROGENASE

Part C. Intracellular Location of Lactate Dehydrogenase

Biological Role of DNA and RNA

DNA is an information molecule with two general functions (Figure 4). First, DNA plays a central role in the propagation of the species and the determination of the heritable characteristics of the cell and its descendants. Prior to the time of each cell division, the two strands of the DNA helix separate from one another and each serves as a pattern or template for the synthesis of a new, complementary chain. This process of DNA biosynthesis is known as replication. One of the double helices formed is then transmitted to one daughter cell, and one to the other. Although the principle underlying DNA replication is straightforward, the actual mechanism responsible for the replication process in the cell involves an array of enzymes and regulatory proteins.

The informational content of DNA also determines the types of proteins that are produced by a cell. In this manner, the DNA molecule functions as a blueprint for all cellular processes that go on during the lifetime of an organism. In the first step along the information pathway from DNA to protein, a segment of DNA is copied into a complementary strand of messenger RNA (mRNA) by a process known as transcription. Transcription begins when an enzyme called RNA polymerase binds to a specific sequence on the DNA known as the promoter. At this site, the enzyme unwinds a small segment of double helix, exposing the bases of the two single strands of the DNA molecule. One of these strands is then transcribed. As the polymerase travels along the DNA, ribonucleotides with bases complementary to the DNA are added to the growing chain. For example, the DNA segment C-G-T-A-T-G is transcribed into G-C-A-U-A-C in the mRNA. Each sequence of three nucleotides in the mRNA is called a "codon," and codes for one amino acid. Since most polypeptide chains contain between 100 to 1,000 amino acids, an mRNA must be at least 300 to 3000 nucleotides long. Therefore, a gene that codes for a polypeptide chain must contain at least 300 to 3,000 base pairs.

The translation of mRNA into protein is a complex process that occurs on particles called ribosomes. This process requires ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs). These RNA species do not specify proteins themselves but rather take part in decoding the information carried by the mRNAs. At one end of each tRNA molecule is a nucleotide triplet called the "anticodon," which is complementary to an mRNA codon. A specific amino acid is bound to the opposite end of each tRNA molecule. On the ribosome, tRNAs carrying amino acids associate with the mRNA by way of complementary base-pairing at the anticodon-codon sequences. As the ribosome moves along the mRNA, the amino acids carried by the tRNAs are linked to the growing polypeptide chain. In this manner, the order of codons along the mRNA directs the amino acid sequence of a polypeptide chain. The translation process occurs on the surface of the ribosomes. These particles, which are composed of rRNA and ribosomal proteins, serve to bring together the mRNAs, the tRNA and other factors that are required for protein production.

Anaerobic metabolism occurs in the absence of oxygen. In the process, pyruvate is converted to 2-carbon or 3-carbon end products which then exit the cell. The process is frequently called fermentation or anaerobic glycolysis and the end products include ethanol, acetic acid, and lactic acid. In organisms such as brewers yeast and some bacteria, pyruvate is converted to ethanol and the process forms the basis of beer and wine production. Pyruvate can also be reduced to lactate in a reaction which is catalyzed by the enzyme lactate dehydrogenase (LDH). This reaction, which is shown below, occurs in most cell types. However, it is especially pronounced in lactic acid producing bacteria, in active muscle cells which frequently display oxygen deficiency during exercise and in a few cell types such as red blood cells, which lack mitochondria..

Lactate Dehydrogenase (LDH)



B. CELL TYPES IN BLOOD

Blood contains erythrocytes, leukocytes, and platelets suspended in a liquid medium called plasma. The erythrocytes, or red blood cells are the most numerous cell type in blood. These cells carry hemoglobin in the circulation and this protein transports oxygen from the lungs to tissues. Mammalian red cells are formed in the bone marrow of the adult by a process called erythropoiesis and then lose their nuclei shortly before they are released into the circulation. In contrast, erythropoiesis in nonmammalian vertebrates does not involve nuclear loss. Thus, erythrocytes in the circulation of adult birds, reptiles, amphibians, and fish contain nuclei.

Mature erythrocytes in all vertebrates lack mitochondria. Thus, complete oxidation of pyruvate to CO₂ and water as in Figure 1-3 does not occur. One major source of energy for the mature erythrocyte is anaerobic glycolysis and a major end product of the process is lactic acid. Thus, lactate dehydrogenase is an important enzyme in energy metabolism in the erythrocyte. In this laboratory, you determine the subcellular location of lactate dehydrogenase in chicken erythrocytes.

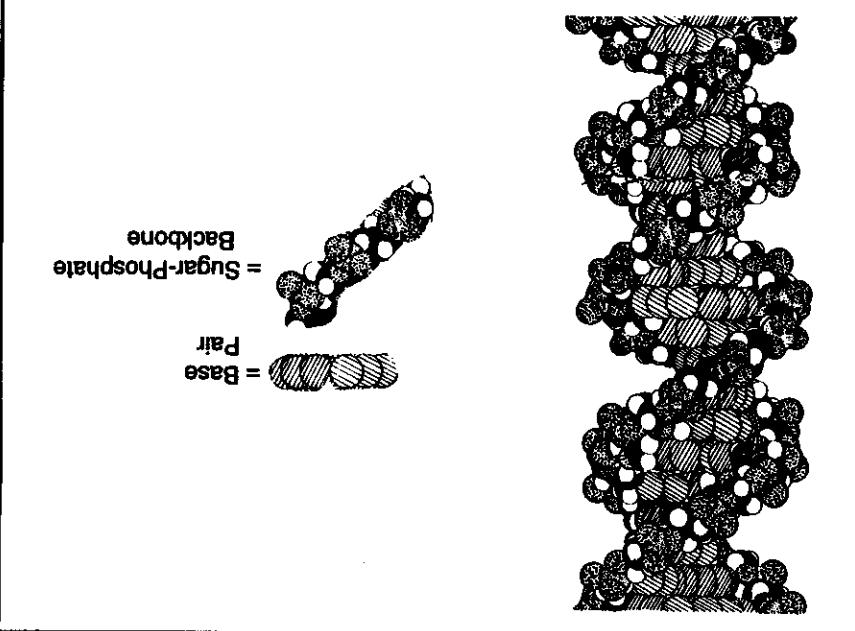
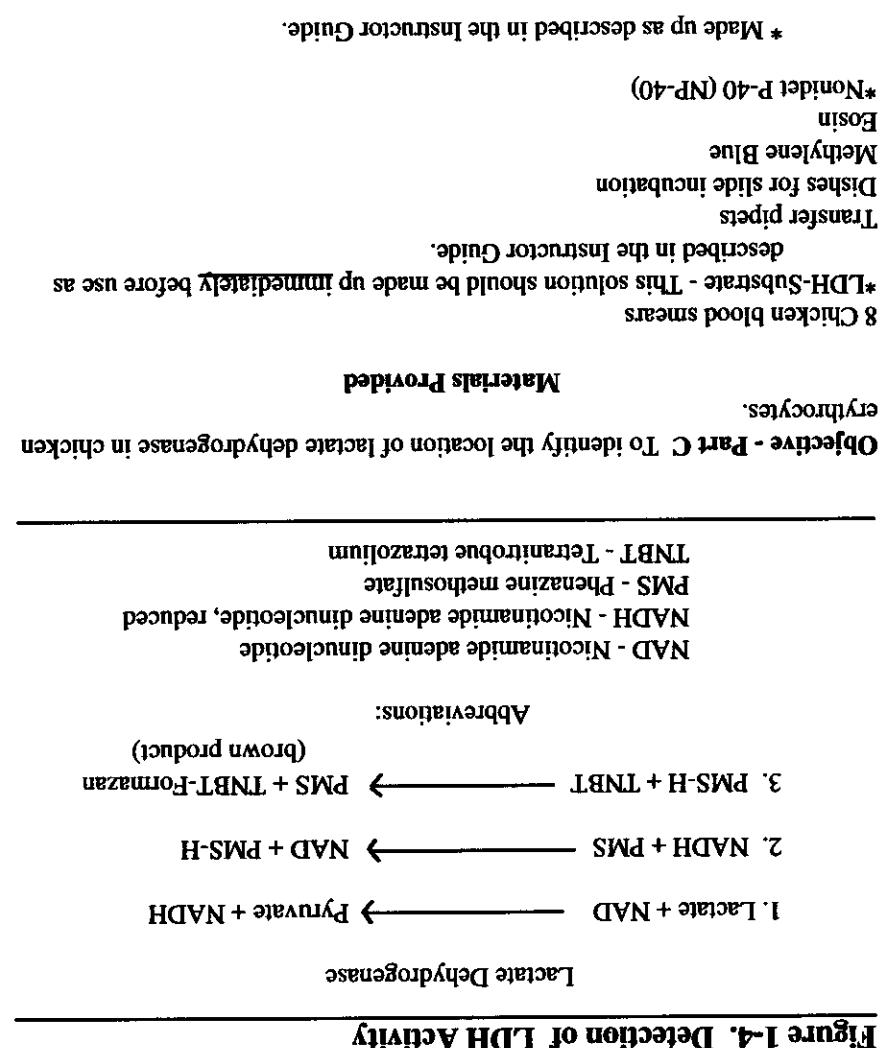


Figure 3. Double-Helical Structure of Common B-DNA

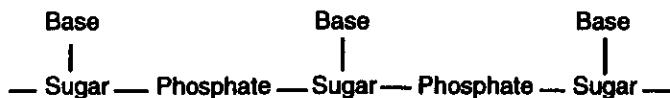
In early physical studies of DNA, a variety of experiments indicated that DNA molecules occur in long helices with each helix being formed from two or more polynucleotide chains bound side by side. Chemical analyses also demonstrated that the phosphate groups were on the outside of the helix and that the number of A and T residues in DNA were always equal, as were those of G and C. With these facts in mind, Watson and Crick in 1953 proposed that the DNA molecule actually consists of two polynucleotide chains coiled around the same axis to form a double helix (Figure 3). In this model, the hydrophilic sugar-phosphate groups follow the outer edges of the molecule where they can interact with water. The hydrophobic bases face inward toward each other in the molecule's center and thus avoid contact with water. The two polynucleotide strands run in opposite directions (they are anti-parallel) and are held together primarily by hydrogen and hydrophobic bonding between the bases, where A is always paired with T, and G with C. These complementary bases have an affinity for each other such that, when they are paired, they contribute to the overall stability of the DNA helix. Because of this complementarity, the sequence of bases in one polynucleotide chain determines the sequence in the other. For example, if the bases along one strand are arranged in the order T-G-C-T-A-G, the opposite bases along the other strand will be A-C-G-A-T-C. This fact is of extreme biological significance because it explains how a DNA helix in the chromosome directs the formation of copies of itself and directs the formation of RNA molecules with its specific informational content. The B-form DNA shown in Figure 3 is the most common of the DNA types. It is a right-handed double helix and contains about 10.5 nucleotide pairs per helical turn.

C. DETECTION OF LDH ACTIVITY



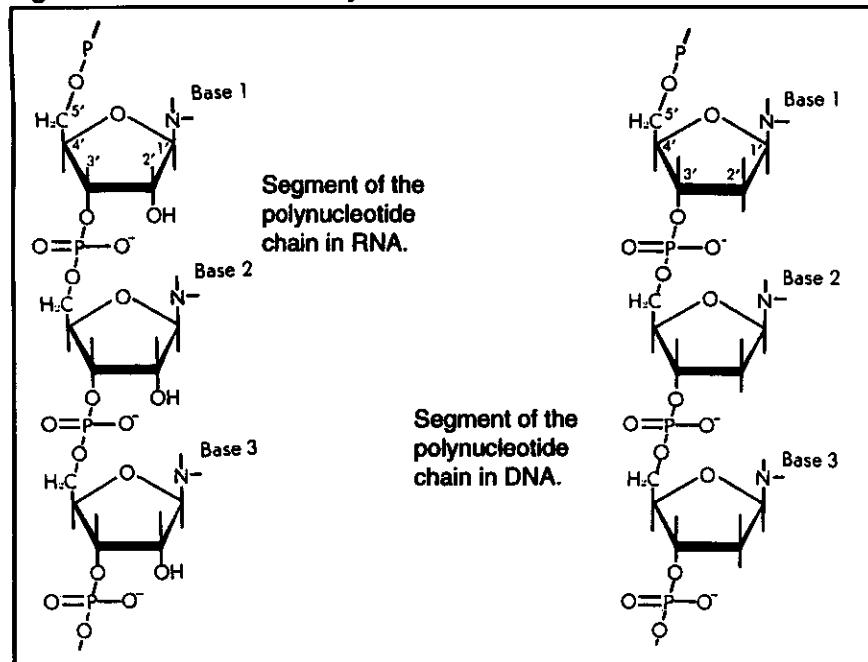
The Polynucleotide Chain

Nucleic acids are polynucleotides and have the general structure shown below:



A polynucleotide is composed of repeating nucleotide units linked into chains by phosphodiester bonds that join the 5' carbon of one ribose or deoxyribose group to the 3' carbon of the next sugar (Figure 2). The sequence or order of nucleotides in a polynucleotide chain is often abbreviated by a 1-letter code (e.g., G-C-A-T-A) with the 5' end of the chain written at the left. A typical RNA molecule is a single-stranded polynucleotide chain. As will be described below, DNA usually contains two polynucleotide strands coiled around one another to form a double-stranded helix. The number of nucleotide units in a nucleic acid chain varies tremendously depending on the nucleic acid type. For example, each chromosome from a higher organism is thought to contain a single, very long DNA molecule. A DNA molecule from the largest human chromosome is composed of approximately 5.4×10^8 nucleotides, which corresponds to a molecular weight of the order of 10^{11} and a length of about 4 cm. On the other hand, transfer RNA molecules generally contain only 70-80 nucleotides.

Figure 2. Structure of the Polynucleotide Chain



Materials Not Provided

Ethyl alcohol (90-100%)
Distilled or deionized water
Microscopes, cover slips, and immersion oil
Small (100ml) beakers
Waterproof markers
Water bath maintained at 37°C (98°F)

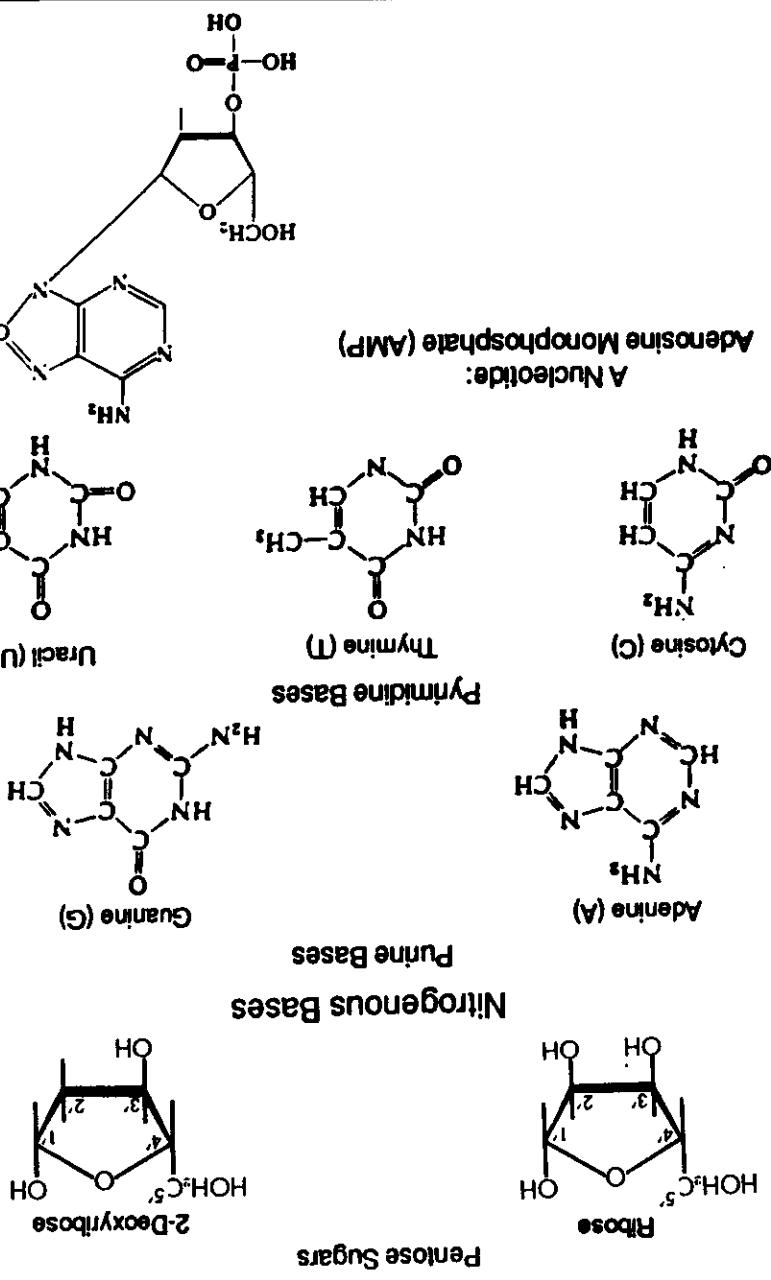
Procedure

I. Fixation

1. Place the slide on the laboratory bench in front of you with the frosted side up. Place your initials on the frosted surface using a pencil. The cells have been spread on this surface of the slide. Place several drops of ethyl alcohol on the slide and after two minutes drain the slide by standing it on end.
2. Allow the slide to dry in air for a few minutes.
3. Rinse the slide with water. To rinse the slide, hold it on the frosted edge at a 90° angle over an empty beaker. Draw a few mls of water into a transfer pipet, slowly expel the water onto the top of the slide near the frosted edge, and permit the water to flow down the slide over the cells. Repeat this process three times.
4. Rinse the slide one time with about 3ml of the detergent NP-40 as described in Step 3. Do not apply the detergent directly onto the cells. The detergent will help to permeabilize the cells which will facilitate the uptake of the LDH substrate.
5. Rinse the slides with water as described in Step 3.

II. Detection of LDH Isoenzymes

1. Preparing the LDH substrate solution: The instructor should prepare the LDH substrate solution. To prepare the solution, transfer the contents of the LDH-Substrate tube to 50ml of distilled water and stir for a few minutes until the powder is dissolved. The solution should be protected from light as much as possible and used immediately after preparation.
2. Place 15ml of the substrate solution into 3 of the plastic dishes. Do not mouth pipet this solution.
3. Place the 8 slides into the three dishes, frosted side up.



- Examine the sections of the slide that were stained with methylene blue and eosin with the low power objective of your microscope and identify the nuclei and cytoplasm of the erythrocytes. Examine several sections of the slide that were not stained by these dyes in order to determine the location of the brown product which is indicative of LDH activity.
- Examine the three regions under oil immersion (if available) with a cover slip or under the highest power objective of your microscope to observe the erythrocytes in more detail. Record the regions of the cells that have stained with methylene blue, eosin and the LDH-substrate.

IV. Microscopy

- Place the slide dry for about five minutes.
- Pipet $\frac{1}{2}$ ml of methylene blue onto the slide about 1 cm away from the end of the slide that is opposite to the frosted end.
- Pipet $\frac{1}{2}$ ml of eosin onto the slide about 2 cm away from the end of the slide that is opposite to the frosted end.
- After about four minutes, rinse the slide with water as described in Section I-3 and air dry for about five minutes.

III. Staining with Methylene Blue and Eosin

- Rinse the slide with water as described in Section I-3 and then drain the slide by standing it on end for about 5 minutes.
- Place the dry slide on the laboratory bench in front of you with the frosted side up and place a ruler next to the slide.
- Place the slide on the laboratory bench in front of you with the frosted side up and place a ruler next to the slide.
- NOTE: Great care should be taken not to bump the dishes during the incubation.

- Place the lids on the dishes and then float the dishes in a water bath at 37°C for 30 minutes.

Part A. Background Information

I. Nucleic Acids: A Review of the Basics

The concept that chromosomal units known as genes transmit heritable information from parent to offspring was founded in the late 19th century. However, a description of genes in terms of their unique structural and functional properties is relatively new. We now know that genes are composed of a type of nucleic acid called deoxyribonucleic acid (DNA). The DNA molecule not only directs its own reproduction but also stores all the information that determines the types of proteins produced during the lifetime of an organism. In so doing, DNA orchestrates the complex reactions and structures characteristic of an organism and its offspring. Ribonucleic acid (RNA), the second major category of nucleic acids, is involved principally in the transmission of genetic information and in protein production. The structure and function of DNA and RNA can most easily be understood by examining the chemical composition of the nucleic acids.

Nucleotides – Building Blocks of Nucleic Acids

Under the proper conditions, nucleic acids can be broken down to low-molecular-weight products of three types: a pentose (or 5 carbon) sugar; purines and pyrimidines; and phosphoric acid (Figure 1). The phosphate group is responsible for the strong negative charge of nucleic acids. The pentose sugar from RNA is always ribose and that from DNA is 2-deoxyribose. These sugars differ only by the presence or absence of a hydroxyl group on carbon 2 (so-called 2'). The numbers assigned to the five carbon atoms are shown in Figure 1. The purines and pyrimidines are often called nitrogenous bases (or, simply, bases). The major purine bases in DNA and RNA are adenine (A) and guanine (G), and the major pyrimidines in DNA are cytosine (C) and thymine (T). RNA contains the base uracil (U) in place of thymine. The sugars and phosphates are readily soluble in water. That is, they are hydrophilic. In contrast, the bases are hydrophobic in that they display limited solubility in water. As will be discussed below, these differences in water solubility are extremely important for the structure of the DNA molecule.

A nucleotide consists of a pentose sugar, a nitrogenous base and a phosphate group structured as shown below. The high-energy storage compound, adenosine triphosphate (ATP), is a well known nucleotide found in biological systems.



Study Questions (For Parts A, B, and C)

1. It has been proposed that peroxidase in plants catalyzes a reaction which serves to strengthen the cell wall. Does the subcellular distribution of peroxidase in onion cells provide support for this view? Explain.
2. What subcellular region of chicken erythrocytes contains the highest concentration of LDH activity? Does this localization of LDH agree with what is known about metabolic processes that occur at this site?
3. Artificial electron acceptors can be used to detect a specific organelle by cytochemistry. Name this organelle and outline a procedure that you would use to detect this organelle by cytochemistry.

Selected References

Seed Physiology, Volume II. "Germination and Reserve Mobilization", D.R. Murray (ed.). Academic Press, 1984.

Molecular and Physiological Aspects of Plant Peroxidases, H. Greppin, C. Pend, and T. Gaspar (ed.) University of Geneva, Switzerland, 1986.

5% acetic acid.

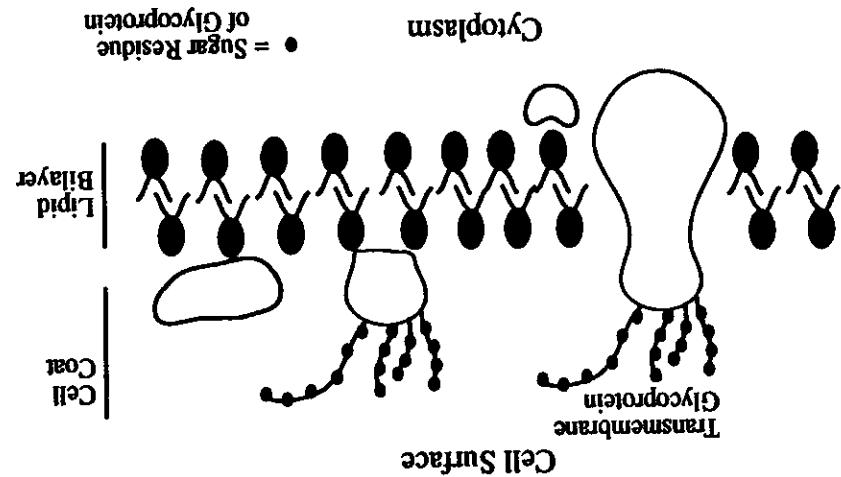
4. The gels can be saved in standard destain solution containing 10% methanol and

2. Add 20mL of the Peroxidase substrate solution to a gel staining dish containing your gel and incubate the dish in the dark for about 30 minutes at 37°C. Do not mouth pipet this solution. Examine the gel every 5 minutes for up to 30 minutes for the appearance of purple bands in lanes containing the Peroxidase standards (lanes 7 and 8) and your tissue samples (lanes 1, 2, 5, and 6). Rinse the gels with water and examine them on a light box.

The instuctor should prepare the subsrate solution at the end of the electrophoretic run. To prepare the solution, add the following to 80ml of water:
 1. 0.35ml of 4M Tris buffer
 2. 3ml of chloromaphthol
 3. 300μl of hydralogen peroxide

IV. Determining Peroxidase Isoenzymes.

The membrane consists of proteins embedded in a lipid bilayer. Most membrane proteins extend across the bilayer (transmembrane proteins) and contain bound oligosaccharide chains (glycoproteins). Less common are oligosaccharides bound to lipids (glycolipids), surface proteins that lack oligosaccharides, and proteins that do not traverse the membrane. Note that the sugar residues of glycoproteins and glycolipids are located exclusively on the outside of the membrane.



ame

The plasma membrane of a eukaryotic cell typically consists of 50% protein, 40% lipid, and 2-10% carbohydrate arranged as illustrated in Figure 2-1. The lipids form a continuous double layer (a bilayer) which inhibits the flow of most water soluble molecules. Some proteins extend across the bilayer and are exposed to both external and internal surfaces of the membrane. These transmembrane proteins are the most common type found in the plasma membrane. Less common are the membrane carbohydrates, which are in the form of glycoproteins that are bound to proteins on the cell surface to form a layer of glycoproteins that is referred to as the glycocalyx. The entire membrane is a dynamic structure with protein and lipid components possessing the ability to move about in a semi-fluid medium.

A. STRUCTURE OF THE CELL MEMBRANE

Background Information

Experiment 2(702). *Analyses of a Cell-Surface Receptor*

III. Electrophoresis

The class should be divided into eight groups for the analysis of the samples by electrophoresis. The samples of each group will be applied to 4 lanes of one agarose gel and two groups will share one gel.

1. Obtain two tubes, label one "Vegetable Extract I" and one "Vegetable Extract II", and add 15 μ l of electrophoresis sample buffer to each.
2. Add 15 μ l of the corresponding vegetable extract to each tube.
3. Load 15 μ l of the following samples into the agarose gel sample wells.

Sample Well Number	Sample	
1	Vegetable Extract I	Group 1
2	Vegetable Extract II	
3	Hemoglobin-Albumin	
4	Cytochrome C	
5	Vegetable Extract I	Group 2
6	Vegetable Extract II	
7	HRP-Basic	
8	HRP-Mixture	

4. Electrophoresis at 170V until the bromophenol blue in the vegetable extract samples has migrated to within 1mm of the (red) positive electrode end of the gel. At 170V, this should take about 30 minutes.

5. Remove the gels from the electrophoresis cell, rinse them in distilled water, and note the position of the colored standard proteins in the gel. In the space provided below, record the distance (in mm) and direction that each protein migrated

Distance from Sample Well (mm)	Direction (Toward the positive or negative electrode)
--------------------------------	---

Hemoglobin (lane 3)	_____	_____
Albumin (lane 3)	_____	_____
Cytochrome C (lane 4)	_____	_____

B. CELL-SURFACE RECEPTORS

Cell-surface receptors are an important class of membrane proteins that are involved in chemical signaling between cells. We shall first consider the receptor for the protein hormone insulin to illustrate a few properties of this class of molecules (Figure 2-2). Insulin is produced by the pancreas and a lack of this hormone causes diabetes mellitus. Insulin stimulates a variety of processes in many cells including an increase in glucose transport and protein synthesis. The cells that respond to insulin contain insulin receptors on their plasma membranes and the insulin receptor is a transmembrane glycoprotein. An increase in blood glucose levels stimulates the pancreas to release insulin, and the hormone travels through the circulation and is recognized by cells containing the insulin receptor. Here, insulin binds to the hormone binding domain of the receptor which is localized on the surface of insulin responsive cells. The binding of insulin to the receptor is a specific interaction analogous to the binding of an enzyme to its substrate. As a result of the binding, the receptor is activated and undergoes a structural change which is transmitted through the membrane to the effector domain of the receptor located on the cytoplasmic side. The effector domain of the insulin receptor is an enzyme (a tyrosine kinase) that is catalytically active only in the presence of insulin. The activated receptor then causes a cascade of events within the cell, which ultimately leads to an increase in glucose transport and protein synthesis.

Figure 2-2. Schematic Diagram of the Insulin Receptor in the Plasma Membrane.

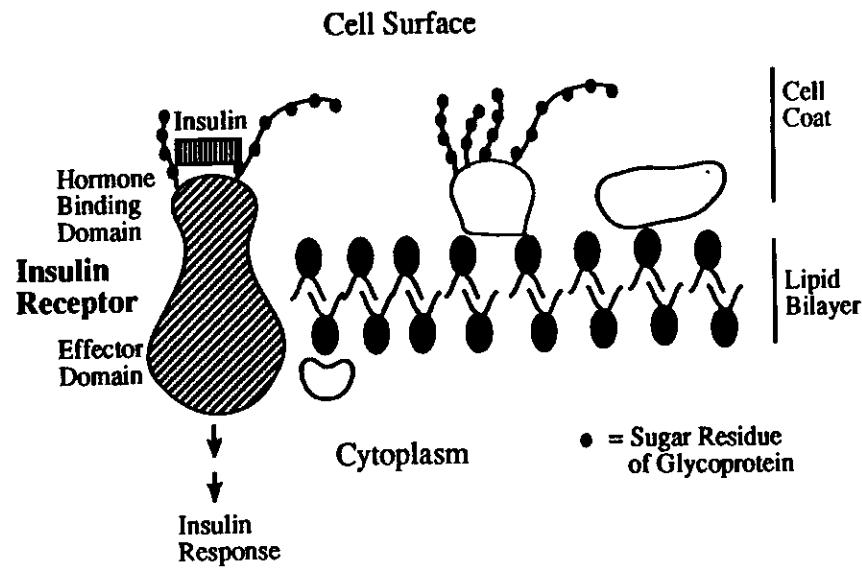


Table 2-1. Properties of a Few Elements

The membranes of human red cells contain two glycoproteins called A and B that form the basis of common blood typing procedures. When red cells containing the A glycoprotein (Type A blood) are mixed with antibodies against this protein, the cells aggregate (stick together to form clumps). Agglutination reactions are induced not only by antibodies, but also by a class of proteins called lectins.

C. LECTURES

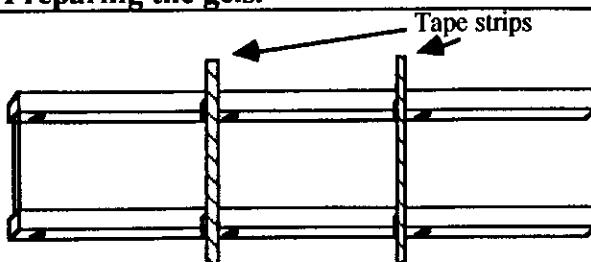
Cell surface receptors are usually involved in numerous types of communication between cells in multicellular organisms. For example, the effects of proteins hormones and serum growth factors in mammals are mediated by membrane receptors. Likewise, the membrane receptors for neurotransmitters play a major role in chemically signaling across synapses in the nervous system. Moreover, a variety of cell-to-cell recognition and communication processes in plants and animals are likely to depend on specific glycoprotein receptors on the cell surface.

1. Place three casting trays, bottom-sides-up, on a laboratory bench and position the trays end-to-end as shown in Figure 4-3. Place two tape strips, 4 1/2 inches long, on the bottom of the trays along the tray junctions and press firmly on the tape to ensure a tight seal.
2. Turn the three trays right side up and press the ends of the tape strips onto the sides of the casting trays.
3. Insert two glass slides into the gel support decks and position the slides such that a pair of combs slots is in the middle of each slide. Place tape strips over both ends of the unit and press firmly to effect a seal.

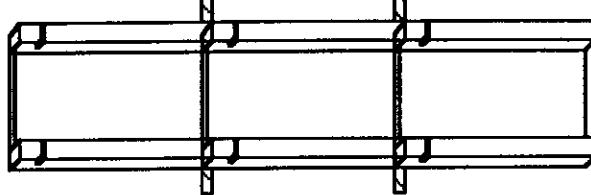
center of the gel. The procedure described on page 54 and diagrammed in Figure 4-3 is for preparing two such gels using the Procell Unit and casting trays. Four gels are needed for the experiment so the procedure must be performed twice.

Figure 4-3. Preparing the gels.

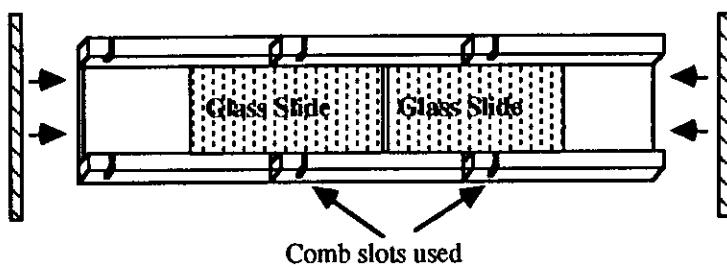
1



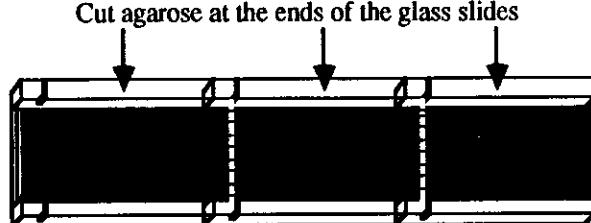
2



3

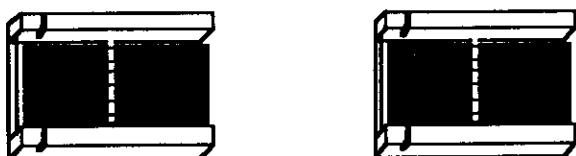


4



Agarose gels with sample wells in the center.

5



* If five casting trays are available, the four gels can be poured at one time. In this procedure, the five trays are taped end to end and 85ml of agarose are poured into the unit.

Lectins are proteins or glycoproteins of nonimmune origin that cause cells to stick or clump together. Over 50 lectins have now been described and the properties of a few of them are listed in Table 2-1. The function of lectins is unclear. Many of the lectins have been isolated from plants whose seeds are a rich source of these interesting molecules. Some seed lectins are toxic when ingested, which may deter animals from eating seeds. However, lectins perform additional functions in plants and animals, and there is a growing body of evidence which indicates that they play roles in cell-signaling and specific cell-to-cell interactions. Thus far, lectins have been implicated in pollen-stigma interactions in some flowering plants, lymphocyte homing mechanisms in mammals, and growth control of cells in culture.

D. LECTIN RECEPTORS

The glycoproteins on the cell surface are composed of proteins covalently bound to oligosaccharide chains (see Figure 2-1). The oligosaccharide chains usually contain fewer than 15 sugar residues and are composed of glucose, mannose, galactose, fucose, galactosamine, galactosamine, and sialic acid. The receptors for lectins are cell-surface glycoproteins and the lectins bind specifically to the oligosaccharide chains. Different lectins bind to different sugar residues and hence bind to different cell-surface glycoproteins. For example, the lectin from the jequirity bean binds galactose and binds to cell-surface glycoproteins containing galactose chains. Pea lectin, in contrast, has a high affinity for mannose and will interact selectively with mannose chains on membrane glycoproteins. This specificity is illustrated in the diagram in Figure 2-3 which shows that mannose inhibits binding of the pea lectin to the cell surface while galactose inhibits binding of the jequirity bean lectin.

SOL

The pH of the Tris-Glycine Electrophoresis buffer that you will use in this experiment is 8.6. At this pH, hemoglobin, albumin, and the acidic isoenzymes of peroxidase are negatively charged and will migrate to the positive (red) electrode during electrophoresis while cytochrome C and the basic isoenzymes of peroxidase are positively charged and will migrate to the negative (black) electrode. In order to identify both types of isoenzymes, the protein sample wells must be placed in the

1. Preparation of the Agarose Gels

Procedure

- *Materials required
- 8 mortars and pestles
- 8 pair of scissors
- 8 razor blades
- 4 gel staining trays
- A water bath main
- Small centrifuge (1
- Eight 1.5 ml tubes

Materials Not Provided

*Prepared as described in the Instructor Guide.

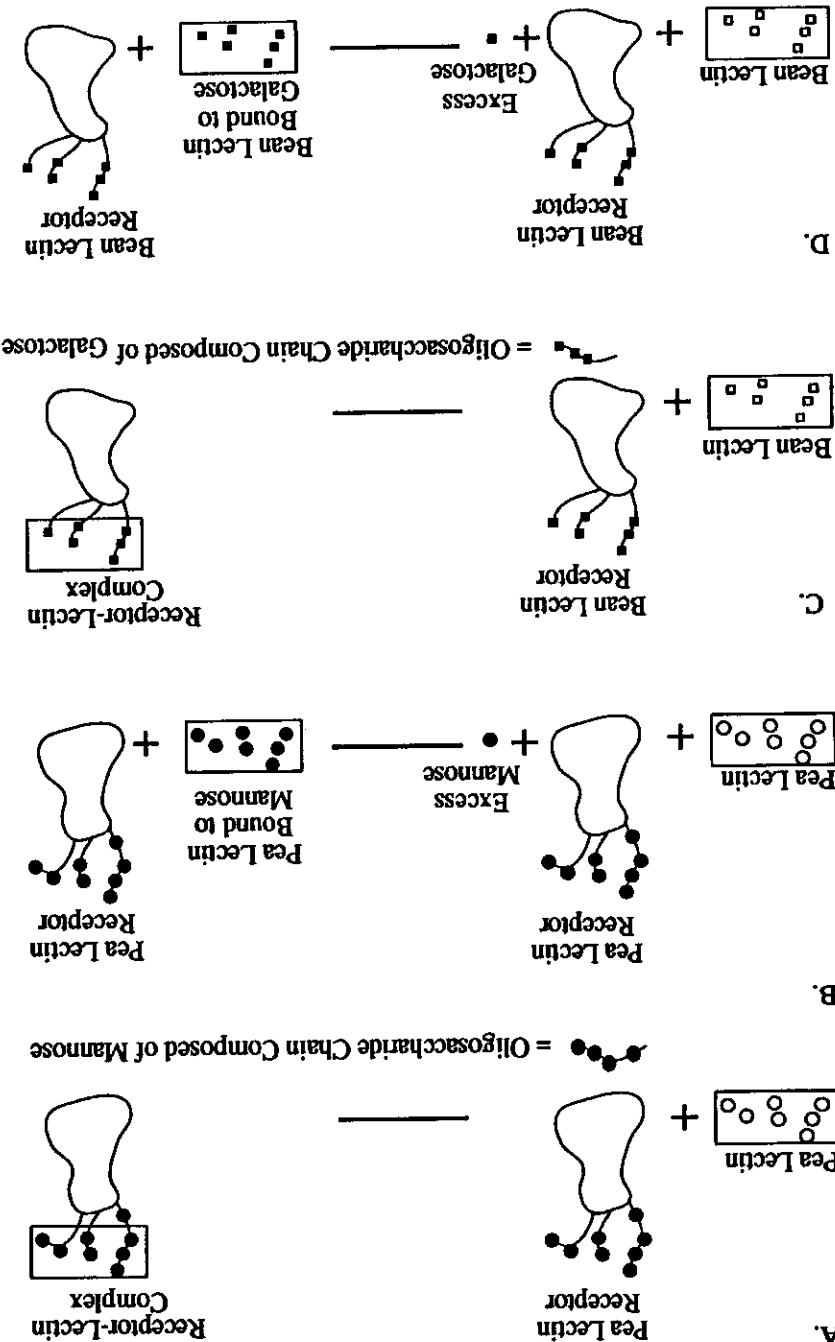
Hemoglobin Substrate Solution - This solution contains hydrogen peroxide, sodium pyrophosphate buffer - The buffer contains NaCl, Tris (pH 8.0) and the detergent Nonidet P-40. *Extraction Buffer - The buffer contains NaCl, Tris (pH 8.0) and the detergent Nonidet P-40.

Peroxidase Substrate Solution - This solution contains hydrogen peroxide, chloronaphthol and its buffer should be made up at the end of the electrophoresis run. Cytochrome C - The protein is dissolved in glycerol and electrophoresis buffer and Hemoglobin-Albumin Mixture - The mixture contains rabbit hemoglobin and cow serum albumin diluted in electrophoresis sample buffer. Upon electrophoresis, the hemoglobin will be visible as a red band. The serum albumin binds bromophenol blue and some of it will remain bound to the albumin during the electrophoresis run.

Vegetable Extracts from the previous laboratory session.
Electrophoresis Sample Buffer - Contains glycerol, electrophoresis buffer, and bromophenol blue.

Materials Provided

Figure 2-3. Binding of Lectins to Cell Surface Receptors.



Cytochrome C - Plant and animal tissues contain a class of cell protein pigments called cytochromes. Cytochrome C, which is one of the most well characterized of the cytochromes, is an integral part of the electron transport system in mitochondria and is involved in cell energy production. Cytochrome C consists of a single polypeptide chain which is wound around a central, nonproteinaceous compound called heme. It is the iron containing heme group which is responsible for the orange-brown color of this protein. The protein is basic in nature primarily because it contains a high concentration of lysine residues. The isoelectric point of horse cytochrome C is 10.2 and at pH 8.6 the protein carries a net positive charge. Thus cytochrome C, unlike most proteins, migrates to the negative electrode during electrophoresis at pH 8.6.

Hemoglobin - Hemoglobin contains an iron containing heme group and the iron is involved in oxygen binding. Hemoglobin is involved in the transport of oxygen in blood. The isoelectric point of hemoglobin from rabbit is 7.2. Thus, this protein should move toward the positive electrode during the electrophoretic separation.

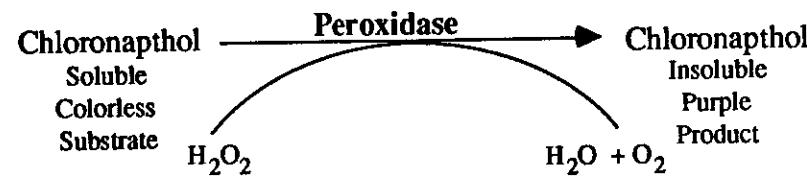
Serum Albumin - Serum albumin is the major protein found in blood plasma. This protein binds and transports a large number of smaller molecules in blood. Unlike the proteins described above, albumin is not naturally colored. However, the tracking dye bromophenol blue has been added to your serum albumin sample and some of this dye will bind and remain bound to the albumin during the electrophoretic run, turning the albumin band blue. The remainder of the bromophenol blue will migrate faster than albumin and when this free dye has migrated to the positive electrode end of the gel, the electrophoretic separation is complete. Serum albumin is a relatively acidic protein and has the lowest isoelectric point of the proteins that will be used in this exercise. Thus, this protein possesses a very negative net charge at pH 8.6, and will migrate faster than the other three proteins described above.

Horse Radish Peroxidase (HRP) - Horse radishes are a rich source of peroxidase and in this laboratory you will use two different preparations as standards. The first preparation (HRP-Basic) contains a single basic peroxidase isoenzyme which will migrate to the negative (black) electrode during electrophoresis. The second preparation (HRP-Mixture) contains three peroxidase isoenzymes: one basic and two acidic.

Lectins are used extensively in the cell biology laboratory to localize, characterize, and isolate specific membrane glycoproteins. In this exercise, you will localize the receptors for a lectin from the Jackbean called concanavalin A (Con A). In order to localize the Con A receptor in cells, a method is needed to visualize Con A by microscopy. One common approach to detect Con A by microscopy is to couple the lectin to a fluorescent dye and then visualize it after cell binding by fluorescence microscopy. An alternative approach is to couple Con A to horse radish peroxidase (HRP) which catalyzes a color producing reaction and this approach will be used in today's experiment.

The procedure that you will use consists of the following basic steps:

1. Cheek epithelial cells are fixed in ethyl alcohol.
 2. The cells are incubated with a Con A-peroxidase complex under conditions which favor binding of Con A to its receptor.
 3. The cells are incubated with substrates of peroxidase (H_2O_2 and chloronaphthol) where the bound enzyme converts the chloronaphthol to an insoluble purple product as shown below.



4. The subcellular site of the purple product is visualized by microscopy.

Objectives In this two-part exercise, you will study the receptor for Con A. In Part A, you will use Con A-peroxidase to determine the location of the Con A receptor in cheek epithelial cells. In Part B, you will study the agglutination of red blood cells induced by Con A. The experiments in both parts will be performed in the presence of various sugars in order to identify the sugar residues on the Con A receptors that are involved in Con A binding.

Laboratory Schedule

Each part of this exercise requires about 90 minutes to complete. The two parts can be performed in the same or in different laboratory sessions. Sufficient materials are provided for 8 groups of students to perform the experiments.

3. Rinse your mouth several times with tap water to remove bacteria and mucus which will interfere with the binding assay.

- Using a pencil, label #1 - #4 on the frosted sections of four clean slides.
- Using a waterproof marking pen, draw a 1cm circle in the center on the bottom surface of each slide.
- Rinse your mouth several times with tap water to remove bacteria and mucus which will interfere with the binding assay.

- The isoelectric point of a protein is defined as the pH at which a protein does not migrate in an electric field - see pages 6-8.
- Bromophenol Blue has been added to the serum albumin sample which stains this protein blue.

Protein	Color	Isoelectric*	Net Charge at pH 8.6	Procedure
Cytochrome C	Orange	10.2	Positive	Immersion oil Immersions (frosted ends preferred) and cover slips
Hemoglobin	Red	7.2	Negative	Small beakers Ethyl alcohol Tooth picks Latex Latex
Serum Albumin**	Blue	4.8	Very Negative	Waterproof markers Small dispensers Ethyl alcohol Tooth picks Latex Latex
Horse Radish Peroxidase (HRP) (Mixture)	Colorless	9.0	Positive	Microtubes (0.5ml or 1.5ml) Waterproof markers
Horse Radish Peroxidase (HRP) (Basic Isoenzyme)	Colorless	9.0	Positive	Microtubes (0.5ml or 1.5ml) Waterproof markers
Positive Negative Negative		6.4		Slide Preparation.

Table 4-1. Properties of the Standard Proteins Used In This Exercise

In order to characterize the peroxidase isoenzymes in the tissue extracts, you will compare their migrations to the migration of our protein standards. The isoelectric points of these are listed in Table 4-1 and a brief description of their functions and properties is given below.

C. PROTEIN STANDARDS

*Con-A-Buffer - The Con A-peroxidase is dissolved in Con A-buffer.

Con A-Buffer - The sugar is dissolved in Con A-buffer.

Galactose (1M) - The sugar is dissolved in Con A-buffer.

Mannose (1M) - The sugar is dissolved in Con A-buffer.

Peroxidase Substrate Solution (Freshly prepared) - This solution containing hydrogen peroxide, chloronaphthol, and Tris buffer should be made up 1-10 minutes before the experiment.

Tranfer pipes

Materials Not Provided

*Prepared as described in the instructor manual.

Microscopes

Clean slides (frosted ends preferred) and cover slips

Small beakers
Ethyl alcohol
Tooth picks
Latex
Latex

Waterproof markers

Microtubes (0.5ml or 1.5ml)

Waterproof markers

Slide Preparation.

The oral cavity is lined by a membrane that is composed of multiple layers of epithelial cells. The cells on the surface of the epithelium are flat (squamous type), and will be used to localize the Con A receptor. Each group of students should prepare 4 slides containing these cells by the procedure described below.

1. Slide Preparation.

2. Using a waterproof marking pen, draw a 1cm circle in the center on the bottom surface of each slide.

3. Rinse your mouth several times with tap water to remove bacteria and mucus which will interfere with the binding assay.

Part A: Location and Properties of the Con A Receptor

Materials Provided

*Con-A-Buffer - The buffer concentrate must be diluted 10 fold with distilled water.

The working buffer contains 0.15M NaCl, 0.1 M Mn SO₄, 0.1M CaCl₂, 0.2% BSA 10, mM Tris-HCl, pH 6.8.

Con-A-Peroxidase - The Con A-peroxidase is dissolved in Con A-buffer.

Galactose (1M) - The sugar is dissolved in Con A-buffer.

Mannose (1M) - The sugar is dissolved in Con A-buffer.

Peroxidase Substrate Solution (Freshly prepared) - This solution containing hydro-

gen peroxide, chloronaphthol, and Tris buffer should be made up 1-10 minutes before the experiment.

Transfer pipes

Materials Not Provided

*Prepared as described in the instructor manual.

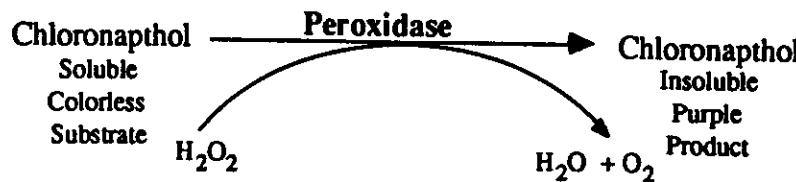
B. PEROXIDASE ISOENZYMES

The enzyme that you will study in today's laboratory is called peroxidase. Peroxidase catalyzes the oxidation of phenolic compounds at the expense of hydrogen peroxide (H_2O_2). Although hundreds of papers have been published on peroxidase, the precise functions of the enzyme are uncertain. In plant systems, peroxidase is likely to play a role in synthesis of the plant cell wall. Here, the enzyme cross links phenolic residues of cell wall polysaccharides and glycoproteins which serve to strengthen the cell wall components. Peroxidase can also kill microorganisms and destroy chemicals that are toxic to both plant and animal cells including H_2O_2 , phenols, and alcohol. For these reasons, it has been proposed that peroxidase protects cells from microorganisms and toxic chemicals.

Isoenzymes are different molecular forms of the same enzyme and different peroxidase isoenzymes are found in different tissues of the corn seedling. Peroxidase isoenzymes have different net charges and thus move differently in an electric field. Some forms of peroxidase are basic proteins and these forms will migrate to the negative (black) electrode during electrophoresis. In contrast, peroxidase isoenzymes which are acidic proteins migrate toward the positive (red) electrode during an electrophoretic run.

In this exercise you will prepare protein extracts from roots and shoots of corn seedlings and then electrophorese the extracts along with protein standards (see below) on agarose gels. The extracts contain hundreds of colorless proteins in addition to peroxidase. In order to identify the peroxidase isoenzymes, you will selectively stain the gels after electrophoresis for peroxidase activity.

Each of the peroxidase isoenzymes can catalyze the following reaction:



4. Using the flattened end of a clean toothpick, gently but firmly scrape the lining of the inside of your cheek to remove a few epithelial cells. Smear the cheek cells on the top surface of slide #1 over the 1cm circle and allow the preparation to dry.
5. Repeat Step 4 three times to prepare slides #2 - #4.
6. To fix the cells, place 1-2 drops of ethyl alcohol on the cells on the top surface of the slides and allow the slides to dry in air for a few minutes.
7. Repeat Step 6 one time. The fixation makes cells permeable to macromolecules and causes them to adhere to the surface of the slide so they will not be washed away during subsequent steps.
8. Rinse each slide with about 2ml of Con A-buffer and stand them on end to remove excess moisture. To rinse the slide, hold the slide on the frosted edge at a 90° angle over an empty beaker with your left thumb and index finger. Draw about 1ml of buffer into a transfer pipet, slowly expel the buffer onto the top of the slide near your thumb, and permit the buffer to flow down the slide over the cells, and into the beaker. Repeat this process one time.

II. The Reaction

1. Obtain four microtubes (0.5ml or 1.5ml) and label them #1 - #4 with a waterproof marking pen.

2. Using a microliter dispenser, add the following to the tubes.

Tube #	Con A-buffer	Con A-Peroxidase	Galactose	Mannose
1	35 μ l	0	0	0
2	10 μ l	25 μ l	0	0
3	0	25 μ l	10 μ l	0
4	0	25 μ l	0	10 μ l

3. Mix the solutions by tapping the tubes with the tip of your index finger.
4. Place the slides on the laboratory bench, top surface up, and dispense 15 μ l of the solution in tube #1 onto slide #1, 15 μ l of the solution in tube #2 onto slide #2, 15 μ l of the solution in tube #3 onto slide #3, and 15 μ l of the solution in tube #4 onto slide #4.
5. After 10 minutes, repeat Step 4.

The top panel shows a diagram of a longitudinal section of a young root. The bottom panel illustrates events at the cellular level that occur in the root zones. In a typical case, a cell divides in the meristematic zone and one of the daughter cells differentiates into a epidermal cell with a root hair.

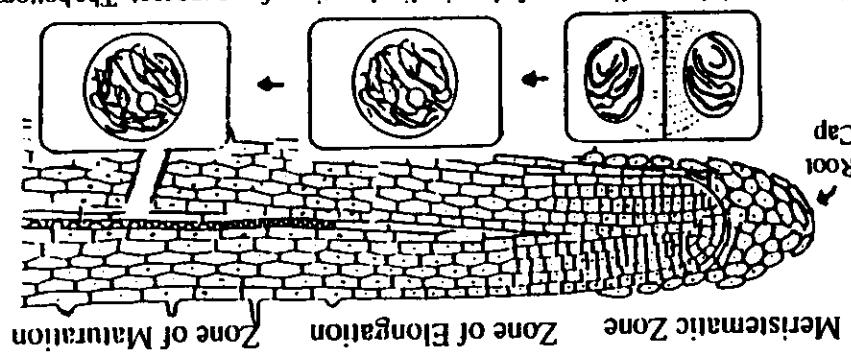


Figure 4-2. Zones of a Typical Young Root.

Numerous biochemical changes occur as the meristematic zone toward the base of the root, Figure 1, begins to differentiate in the root and shoot. Following this development, the apical meristem becomes more organized and produces a primary root system. The primary root system consists of a central axis, the root axis, which bears lateral roots. The primary root system is anchored in the soil by the root axis and provides support for the plant. The primary root system also absorbs water and nutrients from the soil. The primary root system is composed of three main parts: the root cap, the root hair zone, and the root axis. The root cap is a protective layer at the tip of the root. The root hair zone is a region where the root surface is covered with numerous small, hair-like structures called root hairs. The root axis is the main body of the root, which extends downwards through the soil. The primary root system is supported by a network of smaller roots called rootlets, which branch off from the root axis. The primary root system is anchored in the soil by the root axis and provides support for the plant. The primary root system also absorbs water and nutrients from the soil. The primary root system is composed of three main parts: the root cap, the root hair zone, and the root axis. The root cap is a protective layer at the tip of the root. The root hair zone is a region where the root surface is covered with numerous small, hair-like structures called root hairs. The root axis is the main body of the root, which extends downwards through the soil. The primary root system is supported by a network of smaller roots called rootlets, which branch off from the root axis.

metistematac regions of the stem, are densely packed with cell organelles, and are in various stages of the cell cycle. The number of cells in the metistematac zone remains relatively constant, with one daughter remitting in the zone and the other entering the zone of elongation where cell growth and is accompanied by large increases in cell dry weight and protein content. The zone of elongation merges into the zone of maturation, where the elongated cells begin to develop specialized structures and different tissues of the root including the covering layer or epidermis, the cortex and the central vascular cylinder containing the xylem (water carrying cells) and phloem (food carrying elements). Extremally, this zone can be identified by the presence of root hairs which consist of long protrusions of epidermal cells. Growth in length of the root occurs primarily in the zone of elongation, and consequently the metistematac zone is continually moving away from the zone of maturation where cell differentiation is occurring. The metistematac zone is covered by a root cap, consisting of a hood-like mass of cells which protects the meristem as the root grows forward between soil particles.

- A single lectin molecule contains two or more binding sites for receptor molecules. As a result, lectins will form bridges between receptor-containing cells in a reaction. A Con A-induced agglutination reaction with human red cells in order to identify the sugar residues of the Con A receptor that are involved in Con A binding.

Part B. The Con A-Induced Hemagglutination Reaction

Jidde # Purple Color Subcellular Intensity Distribution

In the table below, record the intensity and subcellular distribution of the purple color on your slides.

Data Analysis

- Examine each slide with the low power objective and then under oil immersion to observe the subcellular distribution of the purple color.

4. Prior to microscopic analysis, examine each slide for the intensity of purple color and record your results in the table below.

- After 10 minutes, rinse the slides with water, dry the bottom surfaces of the slides with a paper towel, and stand the slides on end to remove excess moisture.

2. Position the slides on the laboratory bench, frostend side up, and place 3-4 drops of freshly prepared Peroxidase Substrate Solution onto the cells on each slide. After 5 minutes, place additional drops of the substrate solution on the slides.

- Using the washing procedure described in Step 1-8, rinse each slide with about 5 ml of fresh Con-A-buffer. After the last wash, stand the slides on end for about 1 minute to drain excess moisture. This step will remove Con A-peroxidase that is not bound to the epithelial cell.

III. Color Development and Microscopic Analysis

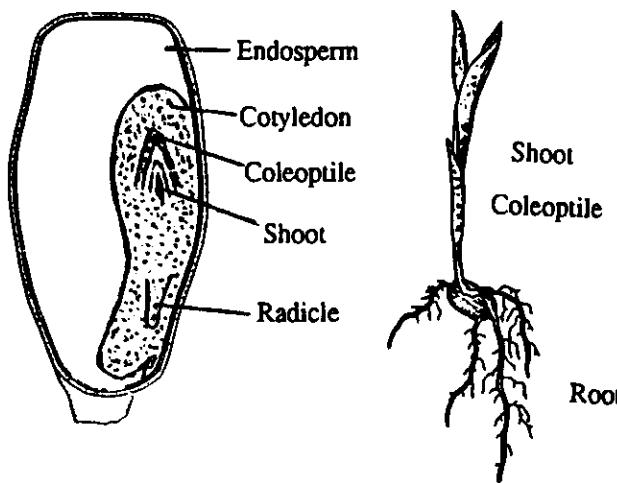
Experiment 4(804) Peroxidase Isoenzymes in Corn

Background Information

A. PLANT DEVELOPMENT

The seeds of flowering plants are typically resistant structures in which embryonic plants are enclosed. The structures in a mature corn seed and corn seedling are shown in Figure 4-1. The mature corn embryo has single cotyledon, or seed leaf, which is made up of a food-adsorbing portion and the coleoptile which forms a protective cap over the shoot. Below the cotyledon is the apex of the embryonic shoot. The basal end of embryo, the radicle, develops into the primary root when the seed germinates. The embryo is embedded in the cellular endosperm, the cells of which are rich in stored protein and especially starch.

Figure 4-1. The Corn Seed and Seedling.



Mature seeds have a low water content and the cells of the embryo are biochemically dormant. The cells can be activated in seeds by environmental factors, especially an increase in the moisture content of the atmosphere. This activation is called germination which begins by the uptake of water (imbibition phase) and culminates in the protrusion of the embryonic root from the seed. In corn, germination requires 24-48 hours under ideal conditions.

Different tissues of a seedling arise by a process in which cell division is followed by cell growth and elongation and finally, by cell differentiation. The roots from seedlings have served as model systems for the study of these important processes. Several regions of the young root may be recognized by microscopy as shown in Figure 4-2. Cell division occurs near the tip (apex) of the root in a specialized tissue called a meristem. The cells in this region, like those in the

Materials Provided

*Con A-buffer

Con A dissolved in Con A-buffer

Mannose (1M) - Dissolved in Con A-buffer

Galactose (1M) - Dissolved in Con A-buffer

*Prepared as described in the instructor manual

Materials Not Provided

Microtubes (0.5ml or 1.5ml)

Microscope slides, coverslips

Microliter dispensers

Blood donor*

Sterile finger lancets

70% alcohol

Sterile cotton

I. Preparation of the Erythrocyte Suspension

For this experiment, one student in the class or the instructor must donate 2 drops of blood.* The donor should:

1. Place 2ml of Con A-buffer into a test tube.
2. Disinfect his/her finger with alcohol, allow it to dry, and puncture it with a sterile finger lancet.
3. Allow 2 full drops of blood to fall into the tube.
4. Shake this tube gently and dispense about 0.2ml of the erythrocyte suspension into 8 small tubes. One tube will be used by each of the 8 groups of students.

II. The Hemoagglutination Reaction

1. One member from each of the 8 groups should obtain 4 small tubes and label them #1 - #4 with a marking pen.
2. Using a microliter dispenser, add the following to the tubes. Be sure to mix the erythrocyte suspension immediately before addition.

*Alternatively, each student can donate his/her own blood or 2 drops of blood can be obtained from a rabbit, rat, or mouse.

Type #	Erythrocyte Suspension	Con A	Galactose	Bufler	Mannosae	IV. Detection of Total Proteins
1	15 μl	20 μl	0	0	0	1. After discarding the water from the petri dish, add 15 ml of protein blot stain. This solution (Ponceau S) should stain all proteins on the nitrocellulose membrane red.
2	15 μl	10 μl	10 μl	0	0	2. After 5 minutes, pour off and discard the stain, wash the membrane 3 times with water and note the regions on the membrane that are red. Attempt to identify those regions on the membrane that are red. Wash the blot stained with the color development solution.
3	15 μl	0	10 μl	10 μl	0	3. Mix the solutions by tapping the tubes with the tip of your index finger.
4	15 μl	0	10 μl	0	10 μl	4. Incubate the tubes for 30-45 minutes at room temperature. During this time, the tubes should be shaken gently every 5-10 minutes.
5. Obtain 4 slides, label them #1 - #4, and place 10 μl of the solutions in tubes #1 - #4 onto slides #1 - #4, respectively. Be sure to mix the contents of the tubes before placing the cells on the slides.						
6. Using a dissecting probe or needle, slowly lower a cover slip over the 10 μl drop on each slide.						
7. Examine each slide with the low power objective of your microscope.						
Count 100 erythrocytes and count the number of them that are in contact with other erythrocytes: Record these values below.						
Slide # % Erythrocytes in contact with other erythrocytes						
1						
2						
3						
4						

Diagrams of Tissue Prints

Study Questions

1. One of the simple sugars (galactose or mannose) used in this exercise should have inhibited Con A-peroxidase binding to cheek epithelial cells and the Con A-induced hemagglutination reaction. Name this sugar and describe the mechanism by which it inhibits these effects.
2. Describe the mechanism responsible for the Con-A induced hemagglutinating reaction.
3. Most membrane proteins including the Con-A receptor can be solubilized by treating cell membranes with a non-ionic detergent such as Triton X 100. You are given such a soluble preparation of erythrocyte proteins. Describe a single procedure that could be used to isolate the Con A Receptor from this preparation.

Selected References

Barondes, S.H. Lectins: Their Multiple Endogenous Cellular Factors, *Ann. Rev. Biochem.*, 50:207-231, 1981.

Zick, Y. The Insulin Receptor: Structure and Function, *Critical Reviews in Biochemistry and Molecular Biology*, Vol 24, pp 217-269, 1989.

7. Record, in the Table below, the relative intensities of the blue color produced by the 5 μ l samples of the four peroxidase standards and by the two samples of vegetable extracts.

Relative Blue Color

use +++ = dark blue
use ++ = medium blue
use + = light blue
use 0 = no color

Peroxidase Standard

#1 (0.01 μ g/ml)
#2 (0.1 μ g/ml)
#3 (1 μ g/ml)
#4 (10 μ g/ml)

Vegetable Extract I

10% Extract
100% Extract

Vegetable Extract II

10% Extract
100% Extract

Characterization of Peroxidase in Plants (Student Designed Projects)

- During the next two laboratory sessions, you will carry out a research project of your own design. The projects will center on the enzyme peroxidase which is found in most plant tissues. Your instructor will provide assorted vegetables which can be used for your work. Alternatively, you may acquire specimens of roots and stems from young trees. In either case, you should begin your work with a hypothesis which is a statement of an idea to be tested in the laboratory. Your work in the laboratory should then revolve around providing evidence to test the idea. Each group will compare the enzyme from two different plant sources. During the first laboratory session, you will localize the enzyme at the tissue level using a technique called tissue-blanching. During this session, you will also prepare cell-free extracts from the two plant tissues and determine the amount of peroxidase present in each. During the second laboratory session, you will analyze peroxidase isoenzymes in the extracts prepared from the two sources in order to characterize the enzyme at the molecular level.
9. Using a razor blade, cut the vegetable, plant stem or root to produce a cross-section. Gently blot the cut surface onto a dry paper towel to remove excess liquid.
10. Position the cut surface of the vegetable onto the nitrocellulose and press down firmly for 10 seconds making sure not to move your hand during the process. Note: The cut surface should be applied at one of the 4 positions of the membrane indicated in Figure 5.
11. Remove the vegetable section and then repeat the process with 3 different vegetables or 3 different sections from the same vegetable. Note: Each tissue must be placed on an unoccupied section of the nitrocellulose membrane as indicated in Figure 5.
12. After all tissue prints have been prepared, place the nitrocellulose membrane in a petri dish with distilled water.

III. Detection of Peroxidase

1. Examine sections of plants that you prepared for tissue printing. With the aid of Figures 2 and 3 and a hand lens, (if available), attempt to identify epidermis, cortex, xylem, and phloem.
2. Examine the tissue prints to determine if impurities of these tissues can be seen on the nitrocellulose membranes.
3. Discard the water in the petri dish and place about 15 ml of freshly prepared color development solution into the dish.
4. Observe development of blue color (peroxidase activity) over the next 5 minutes.
5. After about 5 minutes discard the color development solution and add water to the petri dish.
6. In the space provided on the following page, draw diagrams of the tissue prints. On the diagrams, identify the tissues that contain peroxidase.

IND-2. TISSUE PRINTING

Background Information

A. BASIC ANATOMY OF COMMON VEGETABLES

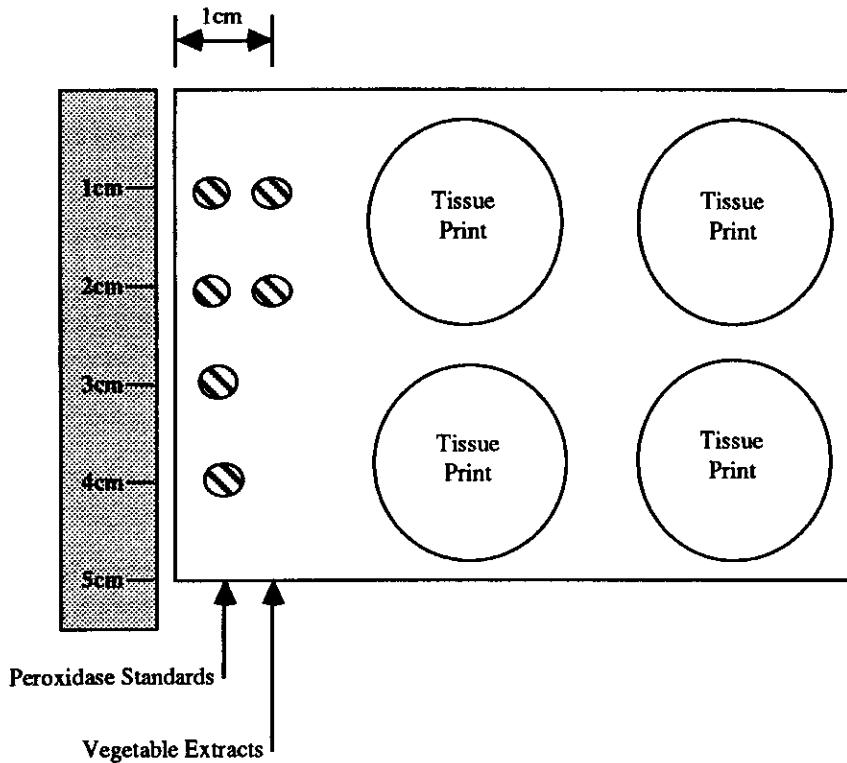
The edible portions of plants are usually divided into fruits and vegetables. Fruits are formed from a matured ovary or from an ovary and associated parts. Examples of fruits include apples, pears, tomatoes, and corn. Any edible part of a plant that is not a fruit is generally called a vegetable. Vegetables can be roots (e.g. carrots, parsnips), normal stems (e.g. asparagus), storage stems or tubers (e.g. potatoes), leaf stalks or petioles (e.g. celery), leaves (e.g. cabbage, lettuce), bulbs (e.g. onions), buds (e.g. brussels sprouts), and flowers (e.g. artichokes). Thus, a basic understanding of plant anatomy and taxonomy is needed for an understanding of the structure of vegetables.

Flowering plants can be subdivided into two groups, the monocotyledons and the dicotyledons. In monocotyledons, the embryo and seedling have one seed leaf or cotyledon. Plants in the lily, palm, grass, and orchid families are examples of monocotyledons. Two seed leaves are found in dicotyledons and this group includes carrots, potatoes, roses, oak trees, and most other herbs and woody plants. Monocotyledons and dicotyledons also differ in the structure of their stems, roots, and leaves and some of these differences are described below.

1. Development and Growth of Plants.

Different tissues of a plant arise by a process in which cell division is followed by cell growth and elongation and finally, by cell differentiation. The roots from seedlings or young bulbs have served as model systems for the study of these important processes. Several regions of the young root may be recognized by microscopy as shown in Figure 1. Cell division occurs near the tip (apex) of the root in a specialized tissue called meristem. The cells in this region, like those in the apical meristematic region of the stem, are in various stages of the cell cycle. The number of cells in the meristematic zone remains relatively constant, with one daughter remaining in the zone and the other entering the zone of elongation where cell elongation occurs. The elongation process involves true cell growth and is accompanied by large increases in cell dry weight and protein content. The zone of elongation merges into the zone of maturation, where the elongated cells begin to develop specialized structures and functions. This process, called cell differentiation, leads to the formation of different tissues of the root including (1) the covering layer or epidermis which protects the soft interior of the root and which absorbs water and dissolved minerals from the soil, (2) the cortex which is chiefly a water and food storage region, and (3) the central vascular cylinder containing the xylem and phloem which serve to conduct water and nutrients through the plant. The xylem forms a continuous system of non living water transport vessels. Water and

Figure 5. Arrangement of Tissue Prints on the Nitrocellulose Membrane.



5. Pipet 5 μ l of each of the four peroxidase standards (#1-4) onto the nitrocellulose about 1/2cm from the ruler. The standards should be carefully pipetted onto the membranes to form individual spots at 1cm, 2cm, 3cm, and 4cm along the edge of the membrane as indicated in Figure 5.
6. Rinse the pipet with water and then pipet 5 μ l of "10% Extract - I" and 5 μ l of "100% Extract - I" onto the membrane to form individual spots at 1cm from the edge of the membrane as indicated in Figure 5.
7. Reapeat Step 6 using "10% Extracts - II" and "100% Extract -II"
8. Allow about 5 minutes for the solutions to be absorbed onto the membranes. During this time, label "Vegetable Extract 100% I and II" with your initials and place the two tubes in the freezer. These extracts

- Procedure**
- Each group of students will use two plant tissues for this project. Both cell-free extracts and tissue-prills will be prepared from these tissues.
1. Prepare one gram of a selected vegetable or plant tissue into a mortar and add 1 ml of enzyme extraction buffer.
 2. Cut the tissue into smaller pieces with scissors.
 3. Grind the tissue with the pestle until a homogeneous suspension is formed.
 4. Wash and dry the mortar and pestle and then repeat steps 1-3 with the second plant tissue to be analyzed.
 5. Transfer the solutions to two centrifuge tubes and label the tubes "Extract I" and "Extract II". Centrifuge the tubes for 5 minutes and remove supernatant fraction (the top liquid) with a clean pipet. Place these solutions into two small tubes and label the tubes "...10% Extract - I or II".
 6. Transfer 5 µl of each of these extracts to two tubes containing 45 µl of distilled water. Label these tubes "...10% Extract - I or II".
- II. Preparation of the Tissue Prints**
1. Wet one sheet of nitrocellulose by floating it in a petri dish with about 20ml of distilled water.
 2. Place one moist paper towel flat on the laboratory bench in front of you.
 3. Remove excess moisture from the membrane by blotting with a dry paper towel.
 4. Place a metric ruler next to the nitrocellulose membrane by holding with a short edge as indicated in Figure 5.
- Note:** Gloves should be worn when handling nitrocellulose to prevent transfer of proteins from your hands to the membrane. If gloves are not available, use forceps. Touch only the edges of the membranes with gloves or forceps.

The growth of the root and stem in length occurs chiefly by the elongation of cells that were produced in the apical meristems. This type of growth is referred to as primary growth and the different tissues that form are called the primary tissues. In most monocotyledons, cells in mature stems and roots are derived from the apical meristems and the issues are primary in origin. However, as the apical meristems grow older, they develop cambium in the root and stem. Cells within the cambium divide and differentiate producing secondary issues which increase the diameter of the plant. Thus, in dicotyledons, most of the issues of the mature root and stem are derived from the cambium rather than from the apical meristems.

The top panel shows a diagram of a longitudinal section of a young root. The bottom panel illustrates events at the cellular level that occur in the root zones. In a typical case, a cell divides in the meristematic zone and one of the daughter cells elongates and then differentiates in the zones of elongation and maturation, respectively.

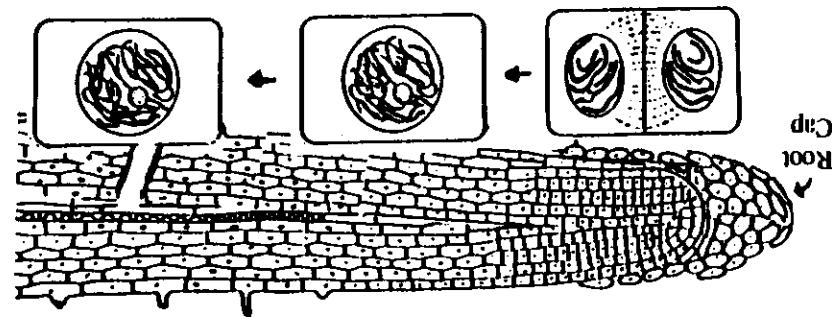


Figure 1. Zones of a Typical Young Root.

Inorganic ions ascend through these vessels in the stem and into the leaves, from which the water is transported (evaporated) into the air. The products of photosynthesis and other organic molecules are transported out of the leaves to other parts of the plant in the phloem.

Objective

To determine the location of peroxidase in selected vegetables by tissue printing and to determine the amount of peroxidase in vegetable cell-free extracts.

Materials Provided

*Peroxidase Standards - The standards contain peroxidase isolated from horse radish. The concentration of peroxidase in the four standards is given below.

Standard Number	Concentration of Horse Radish Peroxidase (μg peroxidase per ml)
1	0.01
2	0.1
3	1
4	10

*Extraction Buffer - The buffer (2mM MgCl₂, 20mM NaCl, 0.01%NP-40, 10mM Tris, pH 8.0) will be used to prepare the vegetable extracts.

*Color Development Solution - Prepared immediately before use by adding 5ml of chloronaphthol, 0.7ml of hydrogen peroxide, and 0.7ml of 4M Tris buffer to 150ml of distilled water.

*Protein "Blot" Stain - Ponceau S

Nitrocellulose (8 sheets)

Transfer Pipets (10)

*Prepared as described in the Instruction Guide.

Materials Needed but not Provided

For Tissue Printing:

Distilled or deionized water

Forceps or gloves for handling the nitrocellulose

Petri dishes

Razor blades

Metric rulers (8)

Paper towels

Hand lens (optional)

Assorted vegetables - Carrots, parsnips, asparagus, and celery are among suitable vegetables for the exercise.

For Preparation and Analysis of Peroxidase in Vegetable Extracts:

One gram pieces of selected vegetables - These may be prepared prior to the laboratory session.

Balance

Scissors (8)

Mortars and pestles (8)

Table-top centrifuge and centrifuge tubes (8)

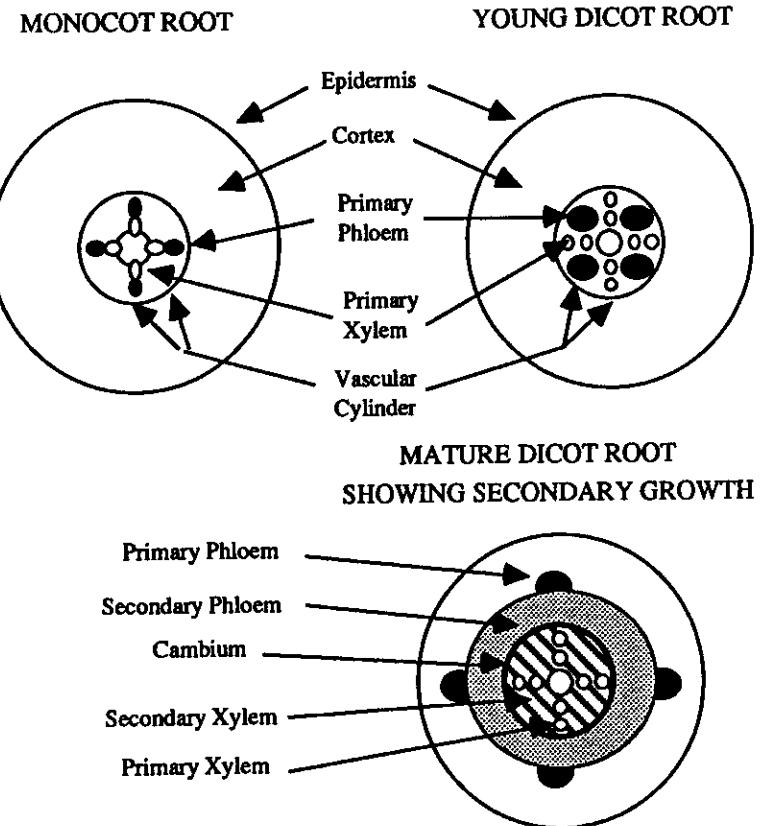
Microliter dispensers (8)

Small (~1.5ml) tubes (16)

2. Structure of Roots and Stems.

There are three major tissue systems in plants: vascular elements, epidermal elements, and packing elements like the parenchyma cells in the root cortex. However, the arrangements of these tissue systems vary in roots, stems, and leaves. In addition, the arrangement is often different in monocotyledons and dicotyledons, and frequently it varies according to the state of the tissue's maturity. Figure 2 shows diagrams of roots from a monocotyledon (monocot) and from a young and mature dicotyledon (dicot). The root from the mature dicot shows secondary xylem and phloem development as is seen in a common carrot. Figure 3 shows diagrams of stems from a monocot and from a young and mature dicot.

Figure 2. Root Structure



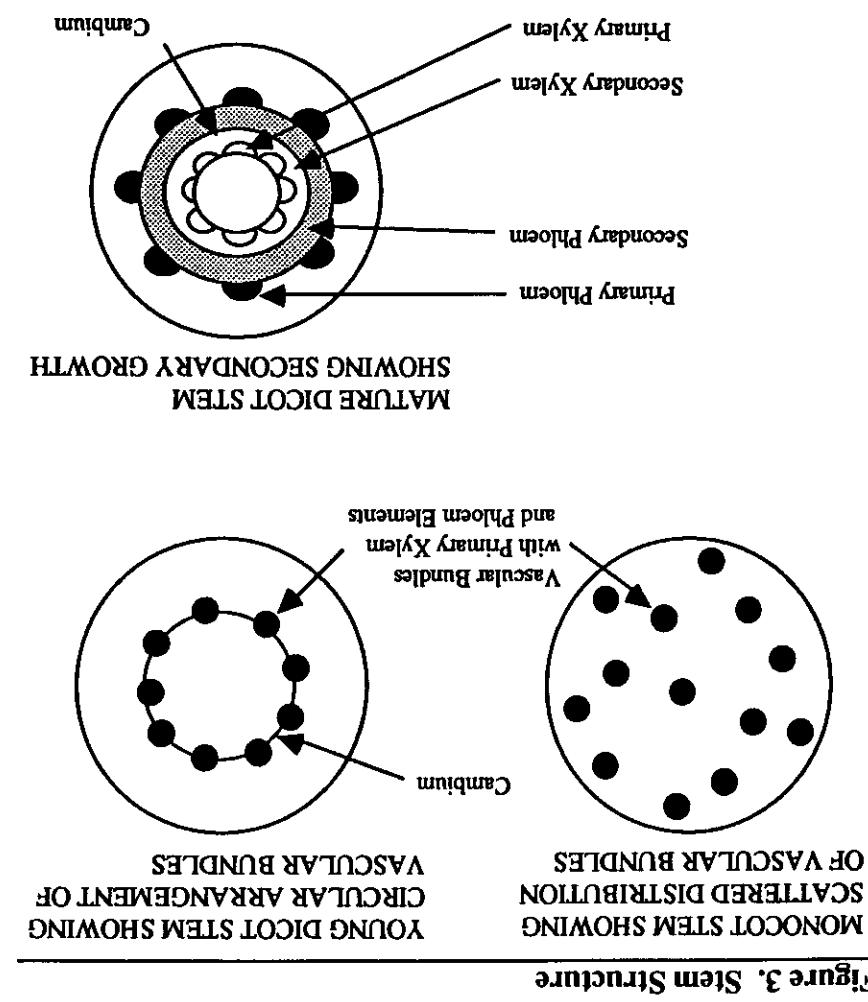
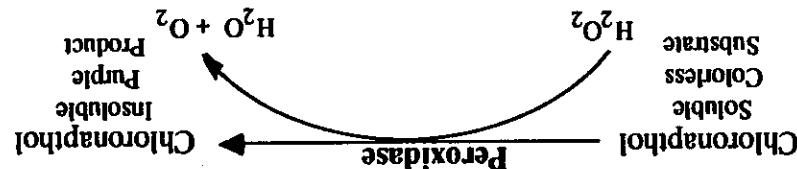


Figure 3. Stem Structure

Figure 4. The Color Development Reaction



In this laboratory, you will localize peroxidase in vegetables by a technique called tissue printing. This technique can be used to localize specific tissue called tissue printing. This technique can be used to localize specific enzymes, antigens, and nucleic acid molecules in animal and plant tissues. You will section vegetables with a razor blade and transfer the proteins from the cut sections to a nitrocellulose membrane by application of gentle pressure. An imprint of the tissue proteins will be formed on the nitrocellulose membrane. The enzyme peroxidase will be detected on the nitrocellulose membrane by incubation with a peroxidase substrate. The peroxidase converts the chloronaphthol to an insoluble purple product which is deposited at the site of the enzyme.

Peroxidase catalyzes the oxidation of phenolic compounds at the expense of hydrogen peroxide (H_2O_2). Although hundreds of papers have been published on peroxidase, the precise functions of the enzyme are uncertain. In plant systems, peroxidase is likely to play a role in synthesis of the plant cell wall. Here, the enzyme cross-links phenolic residues of cell wall polysaccharides and glycoproteins which serves to strengthen the cell wall components. Peroxidase can also kill microorganisms and destroy chemicals that are toxic to both plant and animal cells. Peroxidase protects cells from microorganisms and toxic chemicals.

B. DETECTION OF PEROXIDASE BY TISSUE PRINTING