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Cell Biology Laboratory Manual

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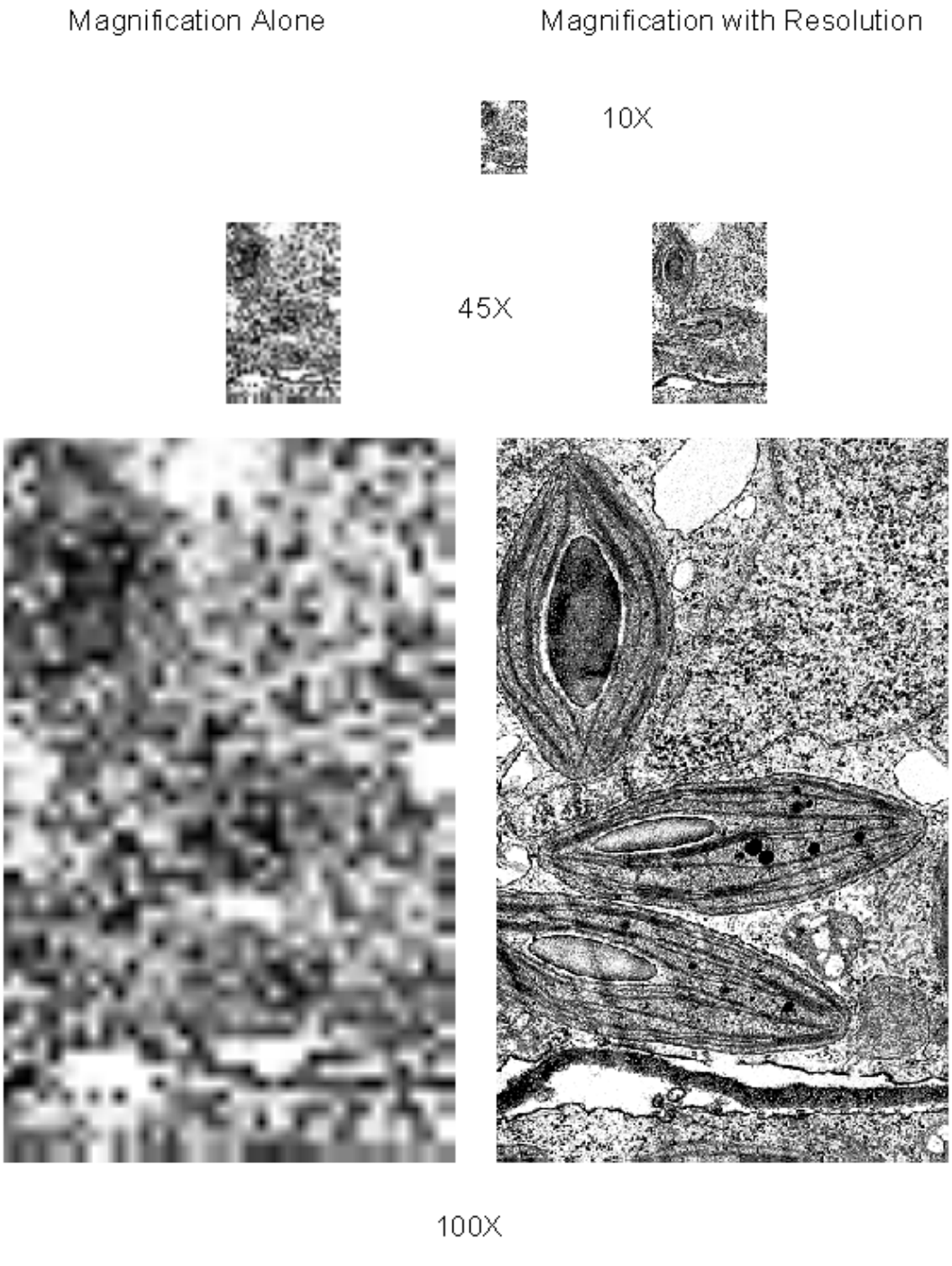
Chapter 1: The Microscope

Introduction

Figure 1.1 Magnification vs. Resolution



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Since its invention, the microscope has been a valuable tool in the development of scientific theory. Magnifying lenses have been known for as long as recorded history, but it was not until the advent of the modern compound light microscope that the device was used in biology. A compound microscope is composed of two elements; a primary magnifying lens and a secondary lens system, similar to a telescope. Light is caused to pass through an object and is then focused by the primary and secondary lens. If the beam of light is replaced by an electron beam, the microscope becomes a transmission electron microscope. If light is bounced off of the object instead of passing through, the light microscope becomes a dissecting scope. If electrons are bounced off of the object in a scanned pattern, the instrument becomes a scanning electron microscope.

The function of any microscope is to enhance **resolution**. The microscope is used to create an enlarged view of an object such that we can observe details not otherwise possible with the human eye. Because of the enlargement, resolution is often confused with **magnification**, which refers to the size of an image. In general, the greater the magnification, the greater the resolution, but this is not always true. There are several practical limitations of lens design which can result in increased magnification without increased resolution.

Figure 1.1 illustrates this point.

If an image of a cell is magnified from 10X to 45X, the image gets larger, but not necessarily any clearer. The image on the left is magnified with no increase in resolution. The image on the right is magnified the same, but with increasing resolution. Note that by the time the image is magnified 10X (from 10X to 100X), the image on the left is completely unusable. The image on the right, however, presents more detailed information. Without resolution, no matter how much the image is magnified, the amount of observable detail is fixed, and regardless of how much you increase the size of the image, no more detail can be seen. At this point, you will have reached the limit of resolution or the resolving power of the lens. This property of the lens is fixed by the design and construction of the lens. To change the resolution, a different lens is often the only answer.

The reason for a dichotomy between magnificatioin and resolution is the ability of the human eye to see two objects. It is necesarry that two objects be about 0.1 mm apart when held 10" from the face in order for us to detect them as two objects. If they are closer than 0.1 mm, we will perceive them as a single object. If two objects are 0.01 mm apart, we can not detect them unless we magnify an image of them by 10X. What has happened is that we have effectively altered our resolution ability from 0.1 mm to 0.01 mm through the use of a magnifying lens. We would say that our limit of resolution has changed from 0.1 mm to 0.01 mm, or inversely, our resolving power (resolution) has increased by a factor of 10.

Unfortunately, a lens can magnify an image without increasing the resolution. Several artifacts can be inherent in the lens design which causes the objects to become blurry at the edges. Thus, even though they can be made to appear 0.1 mm apart, the edges are so blurry that we lose the ability to see them as two objects. Think of a standard eye chart: you can see the increased size of a letter, but may be unable to tell what letter is projected.

Figure 1.1 illustrates what can be seen with increased magnification and resolution. If we were to look only at the left side of the figure, we could get the impression that the cell is filled with a

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homogeneous fluid (cytoplasm). If, however, we look at the right side of the figure, it becomes apparent that the cytoplasm is actually composed of smaller particulate components (chloroplasts, ribosomes, membranes). As we increased the resolution of our microscopes we changed our concepts from protoplasm (the fluid of life) to cytoplasm (the fluid of the cell outside of the nucleus) to a highly ordered machine full of individual organelles.

It is readily apparent that while microscope lenses are usually discussed in terms of their magnification, the most important value is their resolution. All microscopes will come with a lens that can magnify 40 times the normal size, but only a quality lens will allow you to see more than you would with a good hand-held magnifying lens.

As mentioned, the value for resolution may be determined in one of two ways. It can be measured as the smallest distance between two points, which allows us to see the points as distinct. With this measurement, resolution increases as the distance decreases--that is, there is an inverse correlation between the **limit of resolution** and what you actually resolve.

Equation 1.1b

$$\text{Limit of Resolution} = 0.61 \times \lambda / \text{N.A.}$$

To change this to a direct correlation, one need only use the reciprocal of the limit of resolution. **Resolution** is the recriprocal of the **limit of resolution**. For measures of resolution then, as the value increases, resolution increases. Consequently, most microscopists today use resolution rather than limit of resolution to measure the quality of their lenses.

Equation 1.1a

$$\text{Resolution} = \text{N.A.} / 0.61 \times \lambda$$

The resolution of a lens is a property of its physical properties and of the wavelength of light that is passed through the lens. The physical properties are summed up in a value known as the **numerical aperture** while the wavelength is determined by the color of light.

Equation 1.2

$$\text{N.A.} = \theta \times \eta$$

The numerical aperture of a lens is dependent upon two parameters, the **angle of the incidence** of light onto the lens, and the **refractive index** of the glass of which the lens is composed. The angle of incidence is also known as the **cone angle** and 1/2 of this value is designated by the symbol θ. Half the cone angle is used to calculate the angle the light subtends relative to the light axis. The cone angle and thus θ can be altered by inclusion of a substage condenser. If the condenser is moveable, the cone angle can be varied; the closer the substage condenser is to the object, the greater is the cone angle. This is a relatively inexpensive means of effecting the resolution of the microscope and thus nearly all microscopes are equipped with substage condensers.

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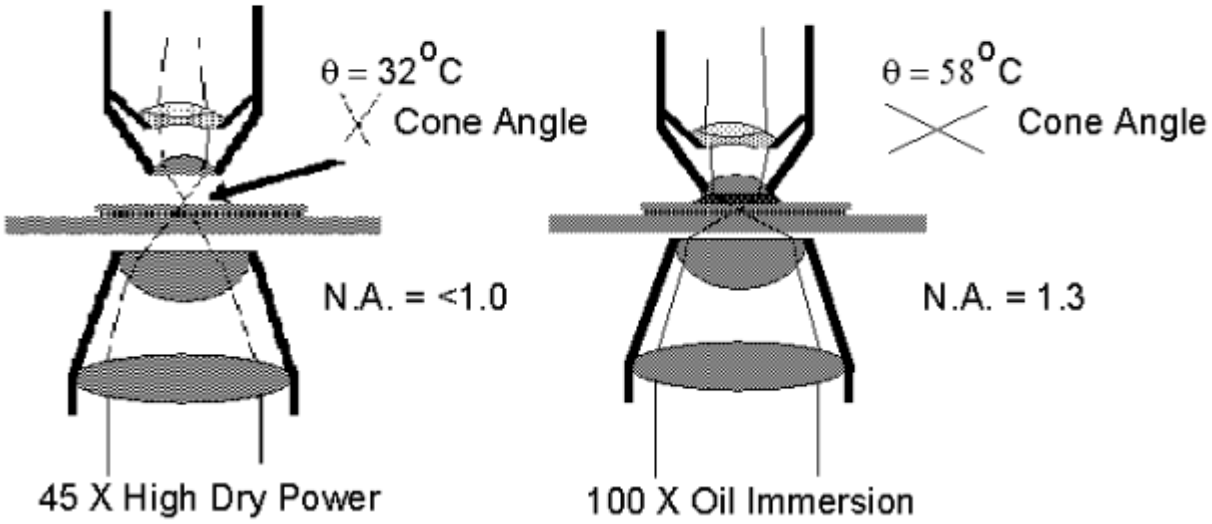
Figure 1.2 Cone Angle and Numerical Aperture



The refractive properties of a lens are summed up in a measurement known as the refractive index (R.I. or η). The refractive index is a function of the bending of light from air through glass and back again. In a microscope, the glass of the lens is specially formulated to increase its refactive index. Once manufactured, however, this property can not be changed. The media around the lens can be altered, however, by removing air from between the objective and the slide, and replacing it with immersion oil. 1

Putting all of this to practical use, it is apparent that resolution can be increased in three ways. The easiest method is to increase the angle of light incidence, by altering the position and/or design of the substage condenser. Second, the refractive index can be maximized by using specially manufactured lenses, and by controlling the medium through which the light travels, i.e. using immersion oil with lenses designed for this purpose. The third method is to decrease the wavelength of light used. For practical purposes, the wavelength has a larger effect on resolution than either changes in the angle of incidence or the refractive index. For maximum resolution, all three properties must be optimized.

For routine bright field microscopy, it is more convenient to work in the visible light range, and the shortest wavelength of visible light is blue. Thus, even inexpensive microscopes have incorporated a blue filter into their design, which is often referred to as a daylight filter. As a rule, the cheaper the microscope the thicker and darker this filter. 2 More expensive and higher quality lenses manipulate the light source to enhance the quality of the light and to correct for lens aberrations inherent in their design.

Resolution can be enhanced by reducing the wavelength to the ultraviolet range and yet again by levels of magnitude to the wavelengths electrons have in motion. The use of electrons as the light

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source gives rise to the electron microscope. UV light can not be seen directly by the human eye (it will injure the retina of the eye) nor can we see electron beams. Thus, these forms of microscopy rely on photography, or upon fluorescent screens.

Visible light ranges in wavelength from the long red waves (λ = 760 nm) to the short blue/violet waves (λ = 400 nm). Ultraviolet waves can be as short as 230 nm. The wavelength of an electron beam depends upon its acceleration voltage, with the wavelength being given by Planck's law. For an electron of charge e, accelerated by a potential difference of V, λ is given by the formula:

Equation 1.3

$$\lambda = h/(2meVe)^{1/2}$$

This is made simpler by the approximation:

Equation 1.4

$$\lambda = (1.5/V)^{1/2} \text{ nm}$$

For an electron microscope with 40,000 volts accelerating voltage, the wavelength of the electron would be 0.006 nm ($(1.5/40{,}000)^{1/2}$). Note that UV light increases the resolution by a factor of 2 over visible light, while the electron microscope has the potential to increase it by a factor of 10^4 to 10^5 over visible light.

Maximum resolution is not attainable, however, unless the lenses are corrected for problems of lens design. Modern microscope lenses are not single lenses, but highly complex collections of lenses assembled to enhance the refractive index while minimizing chromatic and spherical distortions of the image.

Aberrations

Chromatic and Spherical distortions ("aberrations") are inherent in the design of a lens. Because the lens is a sphere, it projects an image that is spherical, while optical theory is based on images that are flat. Moreover, because different wavelengths of light are refracted differently, the spherical image is even further distorted into multiple images, as each wavelength of light forms a separate image.

A lens that is corrected to yield flat fields rather than curved is known as a **plan** lens, while one corrected for flat field and color aberrations is termed a **plan achromat** lens. If the lens is corrected for chromatic aberrations for red and blue, while correcting spherically for green, the lens is an **achromat** lens. Increased resolution and increased cost of the microscope are primary factors in correction of these aberrations. Other than adding colored filters to create monochrome light, there is little or no alteration possible once the system has been built. Thus, we will continue to discuss only those parameters that can be controlled by the user.

Angle of Incidence

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Figure 1.3 Optical path through a light microscope

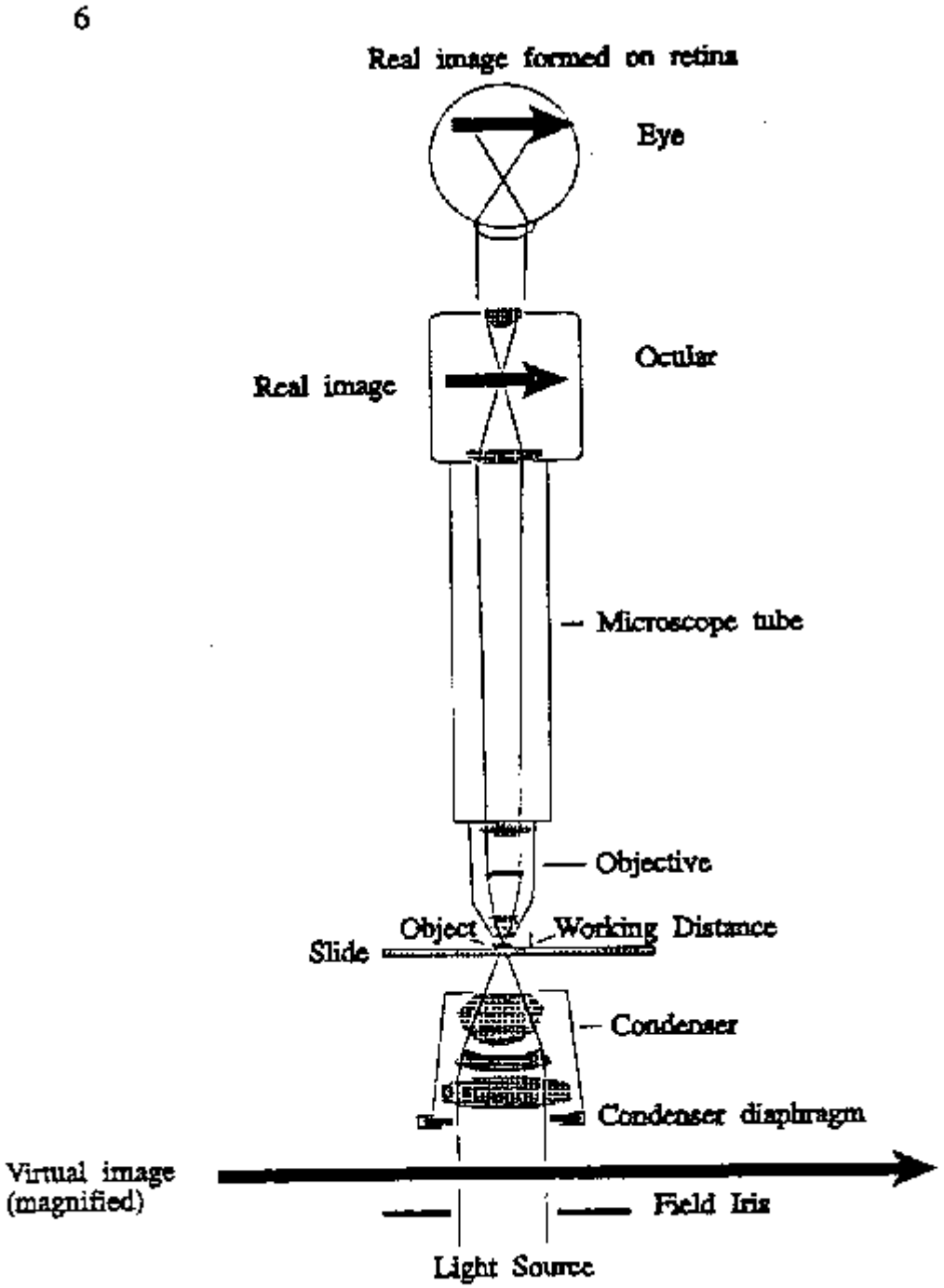

Figure 1.3. Optical path through a light microscope.

Refer to Figure 1.3 for the location of typical microscope components.

While the angle θ can be altered, there is a theoretical limit to this angle which would still allow light to pass into a lens. For any given lens, there should be an ideal or maximum position of the

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substage condenser which would present light to the lens at the appropriate angle, which in turn would allow a maximum light intensity, while maintaining as large as possible. Practically, for most student microscopes at anything above the lowest power (2.5-4X), this is usually in the uppermost position of condenser travel. Good microscopes allow you to see the condenser diaphragm in the field of light and allow precise adjustment of the condenser to its ideal location. Most student microscopes do not allow this, although they will often allow movement of the condenser in a vertical direction, without the ability to adjust the alignment. Since iris diaphragms are inexpensive, virtually all condensers are equipped with these. Iris diaphragms are used to correct for spherical aberrations of the lenses and should be adjusted for each objective. They should not be used to control light intensity, unless resolution is unimportant to the user.

Alignment

Proper use of a microscope demands that the optics and light source be aligned on the optical axis. All of the corrections for aberrations depend on proper alignment of the microscope components. There are two general techniques used for proper alignment of the microscope. The first, and perhaps best, is known as critical illumination. In this process an image of the light source (bulb filament) is projected into the plane of the object, thus superimposing the light source onto the object. It has a distinct disadvantage, however, in that it calls for a flat even light source, not really possible with a tungsten filament bulb.

The second alignment procedure is known as Koehler illumination; after a pioneer in light optics, August Koehler. In this procedure, an image of the field diaphragm is projected onto the object plane. This procedure requires a field condenser lens equipped with a moveable (centerable) iris or diaphragm. Koehler illumination is the most commonly used alignment procedure.

Bright Field, Dark Field, Phase Contrast

All microscopes actually allow visualization of objects through minute shifts in the **wavelength phase** as the light passes through the object. Further image forming can be had through the use of color, or through a complete negative image of the object. If the normal phase shift is increased (usually by 1/4 wavelength), then the microscope becomes a **phase contrast microscope**. Phase contrast microscopes can be designed to have medium phase or dark phase renditions, by altering the degree of additional shift to the wavelength from 1/4 to 1/2 wavelengths, respectively.

If the beam of light is shifted in phase by a variable amount, the system becomes a **differential interference contrast microscope**. The most commonly used system of interference microscopy is known as a Normarski Interference Microscope, named for the optical theoretician, George Nomarski. Once used nearly exclusively by parasitologists, this type of microscopy has increased in use because of the work currently done on the nematode *C. elegans*; interference microscopes are superb for both observation and measuring thickness of embryos within specimens with little or no contrast.

If the light image is reversed, then the microscope becomes a **dark field microscope**. All standard bright field microscopes can be readily converted to dark field by inserting a round opaque disk beneath the condenser. Dark field microscopy was first utilized to examine trans-filterable infectious agents, later to be termed viruses, and to determine that they were particulate in nature.

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Small objects, even those below the limits of resolution, can be detected easily with dark field, as the object appears to emit light on a dark field. Look at the sky for a comparison. It is fairly easy to see stars in a dark sky, but impossible during the day. The same is true for dark field vs bright field microscopy.

Finally, if the normal light microscope is functionally turned upside down, the microscope becomes an **inverted microscope**. This is particularly useful in tissue culture since it allows observation of cells through the bottom of a culture vessel, without opening the container, and without the air interface normally present between the objective and the surface of the culture. By adding phase contrast optics to the inverted microscope, it is possible to monitor tissue cultures directly, without the aid of stains or other enhancements.

The Electron Microscope

The transmission electron microscope (TEM) is the workhorse of histology primarily because of its resolving power (3-10 Å), and its similarity to traditional light microscopy and histotechnique. The scanning electron microscope (SEM) is becoming increasingly popular with cell biologists because of its remarkable ability for quantifiable mapping of surface detail, along with improved resolution (30-100 Å) and its ability to show 3D structure.

The transmission electron microscope is identical in concept to the modern binocular light microscope. It is composed of a light source (in this case an electron source), a substage condenser to focus the electrons on the specimen, and an objective and ocular lens system. In the electron microscope, the ocular lens is replaced with a projection lens, since it projects an image onto a fluorescent screen or a photographic plate. Since the electrons do not pass through glass, they are focused by electro-magnetic fields. Instead of rotating a nose-piece with different fixed lenses, the EM merely changes the current and voltage applied to the electromagnetic lenses.

The size of an electron microscope is dependent upon two factors. Primary is the need for a good vacuum through which the electrons must pass (it takes less than 1 cm of air to completely stop an electron beam). Peripheral pumps and elaborate valves controls are needed to create the vacuum. A substantial electrical potential (voltage) is also needed to accelerate the electrons out of the source. The source is usually a tungsten filament, very much like a light bulb, but with 40-150 Killivolts of accelerating voltage applied to an anode to accelerate the electrons down the microscope column. 3 Modern electronics have produced transformers which are reasonably small but capable of generating 60,000 volts. Transformers used to be room sized (and a large room at that). The million-volt electron microscopes have transformers as large as buildings - in fact, the building housing such a microscope is for the most part a cover for the transformer. While electron microscopes work with high voltage, they use only milliamps of current through the electron gun.

With the need for mechanical stability that is imposed by resolution in the Angstrom range, electron microscopes are usually formidable instruments. Ideally they lie on their own shock mountings to isolate the instrument from building and ground vibrations. The size and cost of an electron microscope is due primarily to the need for stability and maintenance of the vacuum. With advances in vacuum technology (similar to the microchip revolution in electronics), and

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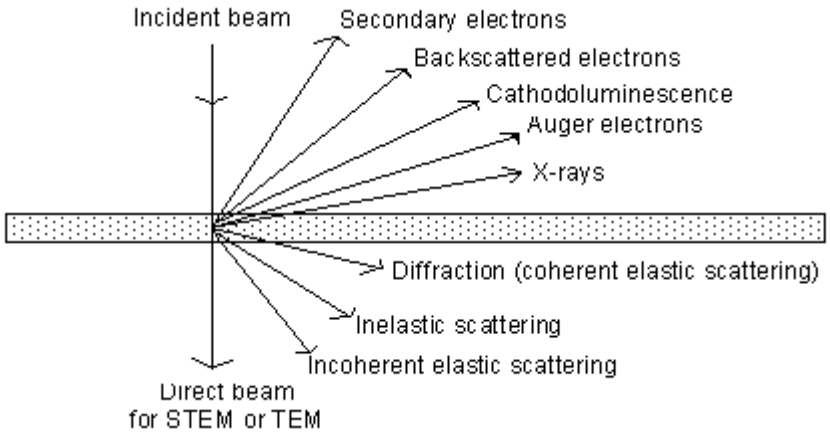
perhaps with the ability to manufacture devices in deep space, the size (and cost) of electron microscopes should decrease.

Another characteristic of electron microscopes is that they are usually designed upside-down, similar to an inverted light microscope. The electron source is on top, and the electrons travel down the tube, opposite to light rays traveling up a microscope tube. This is merely a design feature that allows the operator and technicians ease of access to its various components. The newer electron microscope is beginning to look like a desk with a TV monitor on it.

Until recently, the major advantage of an electron microscope also has been its major disadvantage. In theory, the transmission electron microscope should be capable of giving a resolution of several angstroms. This would give excellent molecular resolution of cell organelles. However, as the resolution increases, the field of view decreases and it becomes increasingly difficult to view the molecular detail within the cell. Electron microscopes designed to yield high resolution have to be compromised to view larger objects. Cell structures fall within the size range that was most problematic for viewing. For example, if we wished to resolve the architecture of an entire eucaryotic chromosome, not just the chromosome, but the cell itself was too large to be seen effectively in an electron microscope. Zooming in on paired chromosomes was impossible. Modern electron microscope design allows for this zooming, and the observation of whole tissues while retaining macromolecular resolution. 4

The Scanning Electron Microscope

Figure 1.4 Useful emission from electron bombardment



The scanning electron microscope works by bouncing electrons off of the surface and forming an image from the reflected electrons. Actually, the electrons reaching the specimen (the 1 ° electrons) are normally not used (although they can form a transmitted image, similar to standard TEM), but they incite a second group of electrons (the 2 ° electrons) to be given off from the very surface of the object. Thus, if a beam of primary electrons is scanned across an object in a raster pattern (similar to a television scan), the object will give off secondary electrons in the same scanned pattern. These electrons are gathered by a positively charged detector, which is scanned in

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synchrony with the emission beam scan. Thus, the name scanning electron microscope, with the image formed by the collection of secondary electrons.

It is possible to focus the primary electrons in exactly the same manner as a TEM. Since the primary electrons can be focused independently of the secondary electrons, two images can be produced simultaneously. Thus, an image of a sectioned material can be superimposed on an image of its surface. The instrument then becomes a STEM, or Scanning-Transmission Electron Microscope. It has the same capabilities of a TEM, with the added benefits of an SEM.

SEM allows a good deal of analytical data to be collected in addition to the formed image. As the primary electrons bombard the surface of an object, they interact with the atoms of the surface to yield even more particles and radiations other than secondary electrons. Among these radiations are Auger electrons, and characteristic X-rays. The X-rays have unique, discreet energy values, characteristic of the atomic structure of the atom from which they emanated. If one collects these X-rays and analyzes their inherent energy, the process becomes Energy Dispersive X-ray Analysis. Combining the scan information from secondary and Auger electrons, together with the qualitative and quantitative X-ray information allows the complete molecular mapping of an object's surface.

Figure 1.4 presents a diagram of the principal emissions from an electron bombardment, while Figure 1.5 compares a secondary electron image to an image recreated from x-ray data.

Finally, the scanning microscope has one further advantage that is useful in cell structure analysis. As the electron beam scans the surface of an object, it can be designed to etch the surface. That is, it can be made to blow apart the outermost atomic layer. As with the emission of characteristic x-rays, the particles can be collected and analyzed with each pass of the electron beam. Thus, the outer layer can be analyzed on the first scan, and subsequently lower layers analyzed with each additional scan. Electrons are relatively small, and the etching can be enhanced by bombarding the surface with ions rather than electrons (the equivalent of bombarding with bowling balls rather than BB's). The resultant Secondary Emmisions-Ion Scanning data can finally be analyzed and the three- dimensional bit-mapped atomic image of an object reconstructed.

Exercise 1.1 - The Bright Field Microscope

LEVEL I

Figure 1.6 Nikon S-Cb

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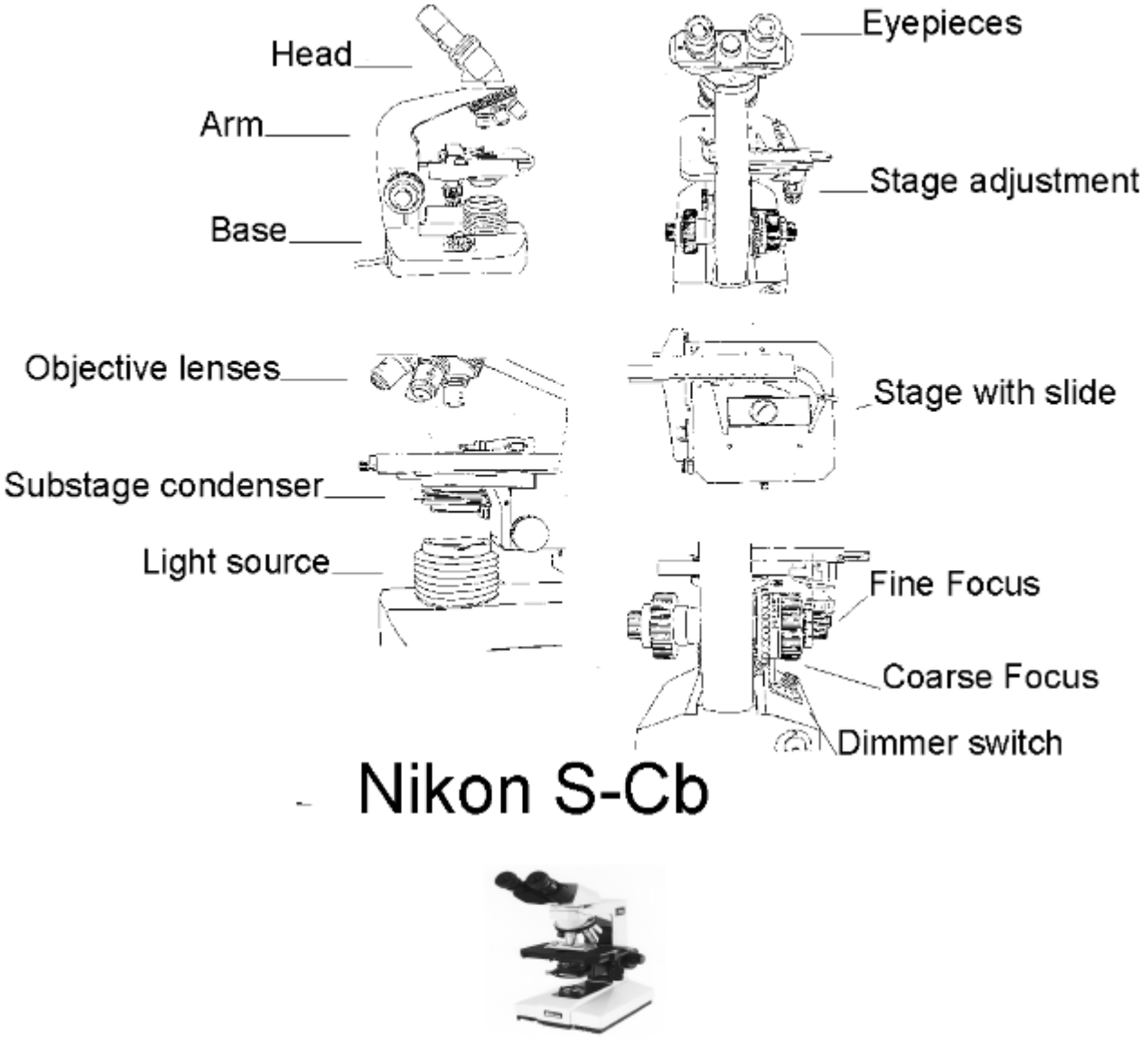

Figure 1.6a Nikon Binocular Microscope

Refer to Figure 1.6 and Figure 1.6a for the names of the various components of a Nikon binocular microscope.

Materials

- Binocular Microscope 5
- Microscope slide with letter e

Procedure

1. Pick up a microscope from the cabinet by placing one hand under the base and the other on the arm of the microscope. Most microscope damage is due to careless transport. It is important that you carry the microscope securely, with two hands, and in an upright position. Remember that you are handling $1,000 of precision instrumentation.
2. Place the microscope in front of you, unwind the power cord and plug it in. The microscope is normally provided in its storage position, that is, with its eyepieces pointed back over the arm. This takes less room in a cabinet, but is not the position for which it was designed to be used. If your instructor

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approves, slightly loosen the screw holding the binocular head and rotate the entire binocular head 180°. Carefully (and gently) tighten the screw to prevent the head from falling off.

You will notice that all parts of the microscope are now conveniently located for your use, with an uninterrupted view of the stage, and substage. The focus controls are conveniently at arms-length.

3. Note the magnification power and the numerical aperture of the lenses which are on your microscope's nose-piece. These values are stamped or painted onto the barrels of the objectives. Record the magnification power and numerical aperture of each lens in the space provided below.

Magnification (x#)	**Numerical Aperture (NA)**

Enter the numerical aperture of the condenser _____

Enter the magnification of the oculars and whether they are normal or widefield _____

Your maximum resolution will depend upon the highest effective numerical aperture of the system. The highest value is normally given by the 100X, or oil immersion lens.

Indicate the numerical aperture of the 100X lens _____

Indicate the numerical aperture of the condenser _____

The numerical aperture for an air interphase = 1.0

The numerical aperture for oil interphase = 1.3 - 1.5

The maximum effective numerical aperture is the lowest of those listed. It depends on the angle and thus on maximum positioning of the condenser. Using the lowest NA value from above as the working numerical aperture, calculate the limit of resolution for your microscope, assuming violet light with a wavelength of 400 nm.

From Equation 1.1b, the limit of resolution = 0.61 x λ/ NA, and therefore, the calculated value for your microscope is:

Limit of resolution = _____ µ

4. Obtain a prepared microscope slide with the letter e. Place the slide on the stage and ensure that it is locked in place with the slide holder.

   Rotate the condenser focusing knob to move the condenser to its highest position of travel. Although there is an ideal location for the condenser, the correct position of the condenser will vary slightly for each objective. Unless directed otherwise, it will not be necessary to move the condenser during any of the intended uses in this course.

   If, however, you wish to find the ideal location, focus the microscope on any portion of a slide, and then

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simply close down the condenser aperture and move the **condenser** until you have a sharply focused view of the condenser aperture (usually with a slight blue hazy edge). If you do this, you can then open the aperture until it just fills the field of view (different for each objective). This is the correct location and use of the condenser and aperture and the condenser should not be moved from this position. Never use the condenser aperture for control of light intensity. Control of light intensity is the purpose of the variable rheostat (dimmer switch, or voltage regulator) on the light source. 6

5. Turn on the microscope by rotating the dimmer switch and adjust the light intensity to a comfortable level. Be sure that the condenser aperture is open if you have not set it as directed in the previous paragraph (slide the condenser diaphragm lever back and forth to check).
6. Looking down into the microscope, adjust the eyepieces to your interpupillary distance and diopter. The Nikon microscope is equipped with a knob between the eye tube extensions for this adjustment. Many microscopes simply require pushing the eye tubes together or apart directly. Move the eye tubes back or forth until you see one uniform field of view.
   The first time you use the microscope, adjust the eyepieces for your personal comfort. Note that modern microscopes have HK (high eye point) eyepieces and consequently you need not remove eyeglasses if you are wearing them. Quite the contrary, they should be worn to prevent eyestrain while you constantly shift from looking through the microscope to reading the lab manual.
   Begin by focusing the microscope on any object within the field of view. 7
   - Find a suitably contrasty location in the center of the field of view and close your left eye. Using the coarse and fine adjustments, focus until you obtain a sharp image with your right eye only!
   - Now close your right eye and adjust the focus of the left eyepiece by rotating the diopter adjusting ring located on the left eyepiece. Do not readjust the focus of the left eye with the coarse or fine adjustments of the microscope - use the adjustment ring on the eye tube.

All subsequent uses of the same microscope will involve use of the coarse and fine focus adjustments, without reference to the procedures in step 2. That is, step 2 need only be performed once at the beginning of your lab. It may, of course, be checked periodically if desired, and will need to be readjusted if someone else uses your microscope.

7. Optional:

   Familiarize yourself with the operation of any tension adjustment options or pre-set devices that may be attached to the microscope. 8

   - Coarse Adjustment Tension: The coarse adjustment may be eased or tightened by the adjusting ring. If the rotation of the coarse focus knob is too loose, turn the adjusting ring counterclockwise. Too much tension may be adjusted by turning clockwise. Avoid excessive rotation as it will place undo stress on the internal gears. Adjust the tension so that the stage will remain stationary after focusing but can be moved with relative ease by turning the coarse adjustment knob. Some microscopes require turning the two coarse adjustment knobs in opposite directions, while others require the use of a screwdriver. Be sure to check with your instructor or the manufacturer's directions before adjusting this feature.
   - Preset Device: On the Nikon S-Cb, the right-hand focus knob has a preset lever on its drum. When the lever is turned clockwise, it will lock the stage so that it can not be moved closer to

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the objective. That is, the stage can be moved away from the objective, but not closer. The preset device is used to rapidly change slides, but has a distinct disadvantage for the neophyte, since it can be locked and the operator might proceed to force the focus. Forcing the focus will result in immediate and costly damage to the microscope!

Unless otherwise instructed, do not use the preset device!

If asked to use the device, refer to the specific directions from the manufacturer. [9]

8. Always begin focusing the microscope with the 10X magnification. Even if you are going to use the 100X, it is more efficient to begin with the 10X and then move up to the power desired. The objective lenses are parfocal, which means that if one is focused, each of the others is approximately in focus when revolved into position.
   With the slide from Step 4 in place, rotate the coarse focus control until the slide is as close to the 10X objective as possible. Move the stage manipulators until a portion of the slide is directly under the objective and focus carefully on the object in view. After adjusting the focus at 10X, center the object to be viewed, and rotate the nosepiece to the next highest magnification. Use the fine focus control only once the 40X or 100X objectives are in place.
   Manipulate the fine focus to obtain the sharpest image. During use of the microscope, one hand should remain on the fine focus as constant readjustment will be called for. Use the other hand to manipulate stage movements.
   Note that the microscope is typically designed so that one revolution of the fine focus knob raises or lowers the microscope stage 0.2 mm. This permits direct readings on the fine focus knob scale to 0.002 mm (2 microns) and can be used to determine the thickness of materials being examined.
9. Return to the 10X objective and move the slide around until you locate the letter e in the view. Note the orientation of the letter e on your slide and in the field of view.
10. To use the 40X objective, center the object you wish to view (the 40X will have a smaller field of view) and rotate the objective turret (referred to as the nosepiece) to bring the 40X objective into position. Is there any change in the orientation of the letter e?

    Do not rotate the turret in such a manner as to bring the 100X into position.

11. Draw the image of the letter e at 10X.

Exercise 1.2 - Use of Oil Immersion (100x)

LEVEL I

Materials

- Microscope equipped with 100X, oil immersion lens
- Immersion oil
- Prepared slide of bacteria or suitably small objects

Procedure

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1. Place the prepared slide on the microscope stage. Using the 10X objective, focus the microscope on an appropriate field containing bacteria. Center the bacteria in the field of view, by manipulating the lateral movement knobs.
2. Rotate the nosepiece to the 40X objective and refocus with the fine focus. Again, center the object which you wish to examine.
3. Rotate the nosepiece so that an intermediate position between the 40X and 100X objectives is obtained.
4. Place a small drop of immersion oil 10 on the center of the viewing area of the slide.
5. Continue to rotate the nosepiece so that the 100X objective is rotated into the oil.

   Do not under any circumstances place the 40x objective in the oil

   Use only the fine focus and refocus your specimen.

6. Determine the shapes of the bacteria and draw them in the place provided.
7. Immediately after using the oil, remove any residual oil from the slide and from the front of the 100X objective by gently rubbing with lens paper dipped in xylol, toluene, or better, a xylol substitute designed for this purpose.

   Nearly all organic solvents, and especially xylol and toluene, are potentially hazardous. They are flammable and readily enter the boddy by direct absorption through the skin or lungs. Consistent high exposure to thses solvents has been linked with liver damage and potential carcinogenesis. Use all solvents sparingly when needed and always in a well- ventilated area.

   Once oil is placed on the slide, the 10X and 40X objectives will no longer be useful until the oil is removed. The oil will blur any image attempted with the lower magnifications. Use oil only when necessary and after completing all work at the lower magnifications. In order to return to work at the lower magnifications, the slide must be completely cleaned of any residual oil and dried. For this reason, oil immersion is employed only when necessary, and only after thorough observations at the high dry magnification (40X). 11

Exercise 1.3 - Measurements: Ocular and Stage Micrometers

LEVEL I

Figure 1.7 Superimposed ocular and stage micrometers

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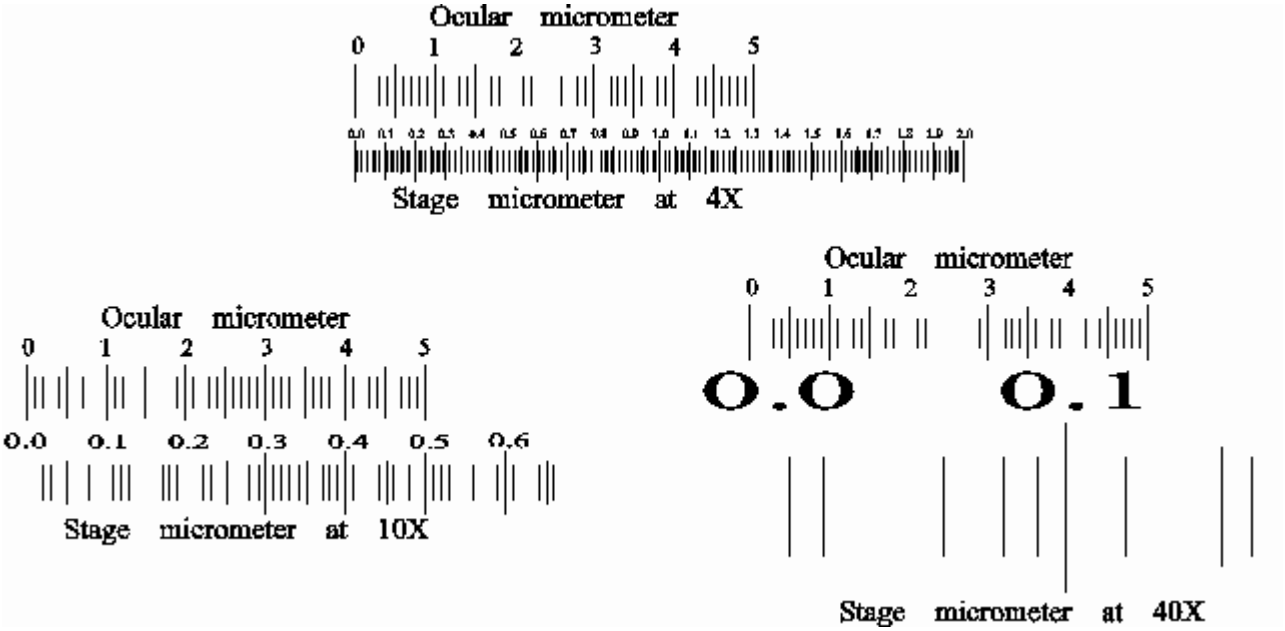

Materials

- Microscope
- Ocular micrometer
- Stage micrometer
- Millimeter ruler
- Prepared slide with letter e

Procedure

1. Place a stage micrometer on the microscope stage, and using the lowest magnification (4X), focus on the grid of the stage micrometer.
2. Rotate the ocular micrometer by turning the appropriate eyepiece. Move the stage until you superimpose the lines of the ocular micrometer upon those of the stage micrometer. With the lines of the two micrometers coinciding at one end of the field, count the spaces of each micrometer to a point at which the lines of the micrometers coincide again (Figure 1.7).
3. Since each division of the stage micrometer measures 10 micrometers, and since you know how many ocular divisions are equivalent to one stage division, you can now calculate the number of micrometers in each space of the ocular scale.
4. Repeat for 10X and 40X, and 100X. Record your calculations below.

Microscope #	
Value for each ocular unit at 4X	
Value for each ocular unit at 10X	
Value for each ocular unit at 40/45X	
Value for each ocular unit at 100X	

5. Using the stage micrometer, determine the smallest length (in microns) which can be resolved with each objective. This is the **measured** limit of resolution for each lens. Compare this value to the theoretical

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limit of resolution calculated on the basis of the numerical aperture of the lens and a wavelength of 450 nm (blue light).

6. Using the calculated values for your ocular micrometer, determine the dimensions of the letter e found on your microscope slide, and add the dimensions to your drawing in Exercise 1.1. Use a millimeter ruler to measure the letter e directly and compare with the calculated values obtained through the microscope.

Notes

To measure an object seen in a microscope, an ocular micrometer serves as a scale or rule. This is simply a disc of glass upon which equally spaced divisions are etched. The rule may be divided into 50 subdivisions, or more rarely 100 subdivisions. To use the ocular micrometer, calibrate it against a fixed and known ruler, the stage micrometer. Stage micrometers also come in varying lengths, but most are 2 mm long and subdivided into 0.01 mm (10 micrometer) lengths. Each objective will need to be calibrated independently. To use, simply superimpose the ocular micrometer onto the stage micrometer and note the relationship of the length of the ocular to the stage micrometer (Refer to Figure 1.7). Note that at different magnifications, the stage micrometer changes, but the ocular micrometer is fixed in dimension. In reality, the stage micrometer is also fixed, and what is changing is the power of the magnification of the objective.

Exercise 1.4 - Measuring Depth

LEVEL I

Materials

- Microscope
- Prepared slide with three colored, crossed threads

Procedure

1. Place a slide containing three colored and crossed threads on the microscope stage.
2. Determine the width (diameter) of the threads using the procedures from Exercise 1.3.
3. Locate a spot where all three threads cross each other at the same point. Use the fine focus control to focus first on the lowermost thread, then the middle thread and finally, the uppermost thread. List the order of the threads from the top to the bottom, by indicating their color.
4. Focus on the top of the uppermost thread. Note the scale markings on the fine focus knob and record the calibrated reading directly from the fine focus control. Carefully rotate the fine focus and stop when the microscope is just focused on the upper edge of the next thread. Record the reading from the focus control below. The difference between the two readings is the depth (thickness) of the upper thread.

Position	Color	Diameter	Depth
Top			

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Middle			
Bottom			

Exercise 1.5 - Measuring Area

LEVEL I

Materials

- Microscope
- Square ocular grid
- Prepared slide of blood smear

Procedure

1. Obtain an ocular grid etched with a square, and insert it into an ocular of the microscope (or use a microsocope previously setup by the instructor).
2. Calibrate the ocular grid in a manner similar to that outlined in Exercise 1.3, and determine the area of each marked grid section. Draw the grid in the space below and add all pertinent dimensions.
3. Place a prepared slide of a blood smear on the microscope and focus on the slide using the 40X objective. Count the number of cells within the four margins of the grid area.
   Count only cells which touch the top and left margins of the grid. Do not count any cell which touches the right or bottom margin of the grid. Record the number in the box on the next page.
4. Select four additional random fields of view with approximately the same density of cells and count the number of cells per grid. Record the numbers in the space provided.
5. Average the results and, based on the known dimensions of your grid, calculate the number of cells per mm^2 for the blood smear. 12

Area of the grid (40X) _____	
Cell Count	**Number of cells/mm^2**
Average number of cells per mm^2 _____	

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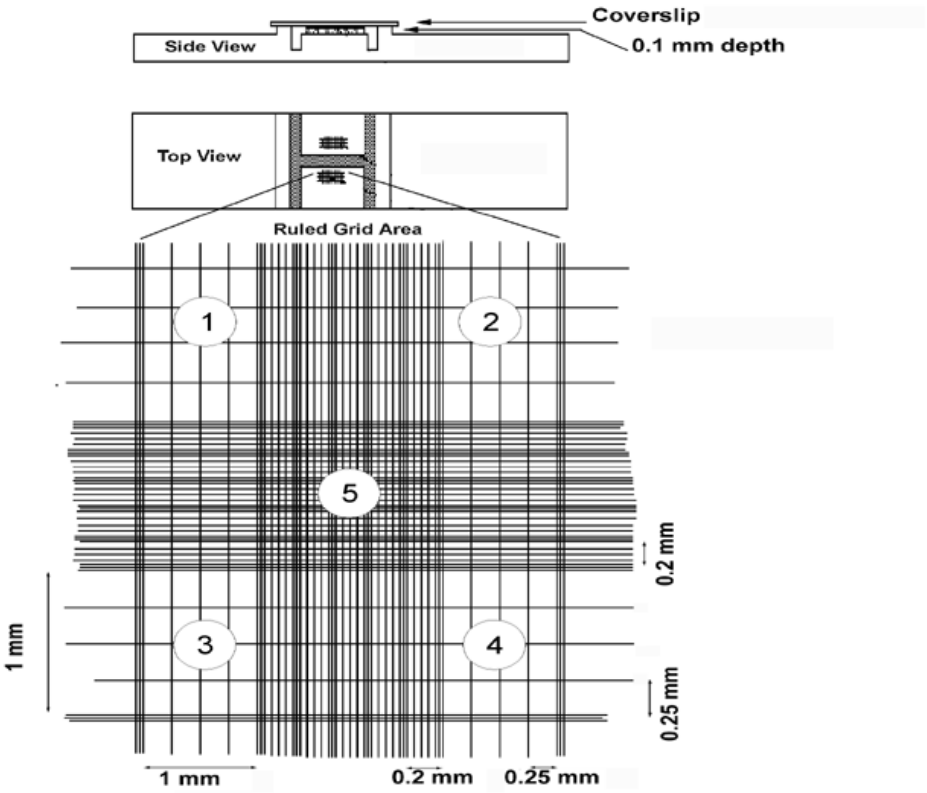
Exercise 1.6 - Measuring Volume

LEVEL I

Figure 1.8 Improved neubauer hemacytometer



Materials

- Microscope
- Hemacytometer and coverslip
- Suspension of yeast 13

Procedure

1. Make a serial dilution series of the yeast suspension, from 1/10 to 1/1000.
2. Obtain a hemacytometer and place it on the desk before you. Place a clean coverslip over the center chamber. 14
3. Starting with the 1/10 dilution, use a pasteur pipette to transfer a small aliquot of the dilution to the hemacytometer. Place the tip of the pipette into the V shaped groove of the hemacytometer and allow the cell suspension to flow into the chamber of the hemacytometer by capillary action until the chamber is filled. Do not overfill the chamber.

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4. Add a similar sample of diluted yeast to the opposite side of the chamber and allow the cells to settle for about 1 minute before counting.
5. Refer to the diagram of the hemacytometer grid in Figure 1.8 and note the following:

   The coverslip is 0.1 mm above the grid, and the lines etched on the grid are at preset dimensions.
   The four outer squares, marked 1-4, each cover a a volume of 10^{-4} ml.
   The inner square, marked as 5, also covers a volume of 10^{-4} ml, but is further subdivided into 25 smaller squares. The volume over each of the 25 smaller squares is 4.0 X 10^{-6} ml.
   Each of the 25 smaller squares is further divided into 16 squares, which are the smallest gradations on the hemacytometer. The volume over these smallest squares is .25 X 1^{-6} ml.
   Given these volumes, the number of cells in a sample can be determined by counting the number of cells in one or more of the squares. Which square to use depends on the size of the object to be counted. Whole cells would use the larger squares, counted with 10X magnification. Isolated mitochondria would be counted in the smallest squares with at least 40X magnification.

   - For the squares marked 1-4, the area of each is 1 mm^2, and the volume is .1 mm^3. Since .1 mm^3 equals 10^{-4} ml, the number of cells/ml = Average # of cells per 1 mm^2 times 10^4 times any sample dilution.
   - For the 25 smaller squares in the center of the grid marked 5, each small square is 0.2 x 0.2 mm^2, and the volume is thus 0.004 mm^3. For small cells, or organelles, the particles/ml equals the Average # of particles per small square times 25 X 10^4 times any sample dilution.
   - Grids 1-5 are all 1 mm^2. Grids 1-4 are divided into 16 smaller squares (0.25 mm on each side), grid 5 is divided into 25 smaller squares (0.2 mm on each side). Grid 5 is further subdivided into 16 of the smallest squares found on the hemacytometer.

   For the yeast suspension, count the number of cells in 5 of the intermediate, smaller squares of the hemacytometer. For statistical validity, the count should be between 10 and 100 cells per square. If the count is higher, clean out the hemacytometer and begin again with step 3, but use the next dilution in the series.
   Record the dilution used, and the five separate counts.

6. Average your counts, multiply by the dilution factor, and calculate the number of cells/ml in the yeast suspension. Record this information in the space provided.

Square #	**Cell Count**
1	
2	
3	
4	
5	
Area of each square = _____ mm^2 x 0.1 mm depth = volume of each square. Volume of each square = _____ mm^3	

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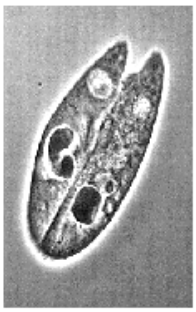
Average number of cells per mm³ = _____
Number of cells per cm³ (1000 x above) = _____
Note: Number of cells per cm³ is also number per ml.
Number of cells per ml _____ x dilution factor (200) = _____ cells per ml of whole blood.

Exercise 1.7 - Measurement of Cell Organelles

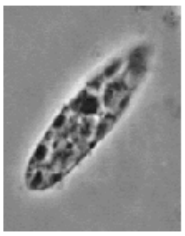
LEVEL I

Figure 1.9 Photo of various cells showing organelles

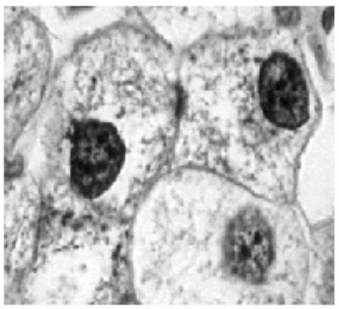
Paramecia



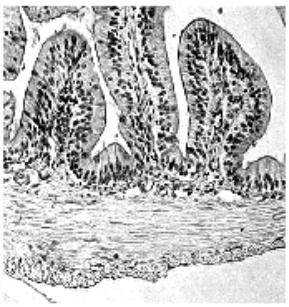
Paramecia conjugation



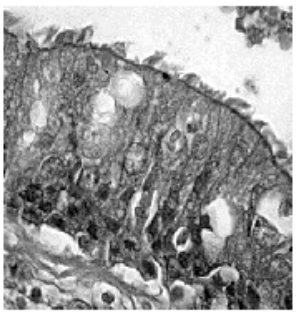
Euglena



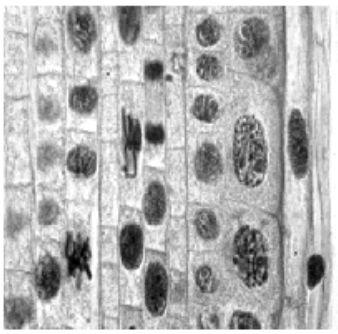
Liver stained for mitochondria



Intestine

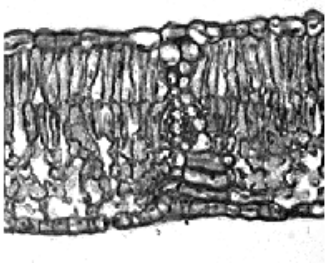


Trachea



Onion Root cells

Capsella embryo



X.S. Dicot leaf

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Materials

- Prepared Slides of the following:
  - Paramecium
  - Euglena
  - Spirogyra
  - Onion Root Tip
  - Trachea
  - Mitochondria
  - Dicot Leaf x.s.
- Microscope

Procedure

1. Each of the slides presents a representative cell, with various organelles of particular interest. Figure 1.9 gives a sample of some of these cell types. Observe each slide, and make a drawing of each cell and its representative organelle(s). Each drawing should meet the following guidelines:
   - All drawings must be completed in pencil. A #3 pencil is preferred since it will not smudge as readily as the standard #2. Colored pencils are optional. Ink is unacceptable.
   - Each drawing must be a minimum of 4" x 5" in size. Smaller drawings will not demonstrate sufficient detail, while much larger drawings require excessive time to fill in detail.
   - Drawings should be completed during the lab period and where applicable, on any forms provided.
   - All labels should be added and appropriate indications of size should be made. Size is best indicated by including a ruler bar at the bottom of the drawing, drawn to scale and with the dimensions added.
2. Measure the appropriate dimensions of all structures. Label the drawings, and indicate the magnification used to view each.
3. On each drawing, place a line (bar) to indicate a length of 10 microns.

Exercise 1.8 - Use of Darkfield Illumination

LEVEL II

Materials

- Microscope with dark field stop
- Suspension of *Amoeba proteus*
- Transfer pipette
- Slides, coverslips

Procedure

1. Make a simple wet mount of the Amoeba by placing a drop of the culture on a slide and placing a cover slip on the slide.
2. Observe and measure the Amoeba using normal bright field optics.

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3. Place a dark field stop 15 in the filter holder below the microscope's substage condenser and and continue to monitor the movements of the amoeba.
4. Note the differences in observable structure possible with dark field microscopy when compared to bright field. Draw and label the amoeba viewed in both ways.

Exercise 1.9 - The Phase Contrast Microscope

LEVEL II

Materials

- Phase contrast microscope
- Telescopic ocular for centering phase rings
- Culture of *Amoeba proteus*
- Transfer pipettes, slides, coverslips
- Prepared, prestained slide of *Amoeba proteus*

Procedure

1. Establish Koehler illumination on the microscope. If the instructor approves, center the phase annular ring and its corresponding phase plate.
    - Place a slide on the stage of the microscope, move the condenser to its highest position and focus at 10X magnification.
    - Open the condenser diaphragm to its maximum setting, and close the field diaphragm completely
    - Using the condenser movement control, move the condenser until a sharp image of the field diaphragm is observed. To determine that the focus is indeed the field diaphragm, slightly open and close the field diaphragm to see if its movement can be detected in the field of view. When focused, there will be a slight blue haze on the edge of the diaphragm.
    - Open the field diaphragm until it nearly fills the field, but can be still seen. Center the field diaphragm in the field of view using the centering screws on the substage condenser. Open the field diaphragm to completely fill the field of view.
    - Remove one of the oculars from its tube and while peering down the tube, open the condenser diaphragm until it just fills the field of view at the bottom of the tube. Replace the ocular in the tube.

      Completing steps a-d establishes Koehler illumination, where the field diaphragm is superimposed onto the object and centers the major optical components of the microscope.

      To check on the phase annulus and its corresponding phase plate, remove an ocular and replace it with a telescopic ocular designed to focus on the rear lens of a phase objective. Match the phase objective with its corresponding setting on the phase condenser and visually verify that the phase annulus (a clear ring) is perfectly matched to the phase plate (a darker ring). If it is not, ask the instructor for assistance in centering the phase annulus. This is most often accomplished by

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adjusting a second set of centering screws attached to the phase condenser. Replace the normal ocular before using the phase contrast optics. Return the phase condenser setting to the normal bright field position.

2. Make a simple wet mount of the amoeba and observe under bright field microscopy at 10X.
3. Locate an active amoeba and center it in the field of view. Rotate the condenser phase ring to match the 10X phase with the 10X objective.
4. Observe the difference in the appearance of the amoeba between normal bright field and phase contrast.
5. Draw the amoeba viewed under phase contrast. Label organelles which are more clearly visible with phase contrast than with bright field microscopy.
6. Return the phase control on the condenser to the normal bright field setting, switch to a higher magnification (20X or 40X) and observe the amoeba at the higher magnifications with and without phase enhancement.
7. Compare the view of the amoeba under phase contrast, normal unstained bright field and darkfield (Exercise 1.8) with the view of the prestained commercial preparation of *Amoeba proteus*. List the organelles and/or structures which are more clearly demonstrated by each optical technique.

Exercise 1.10 - The Inverted Phase Microscope

LEVEL II

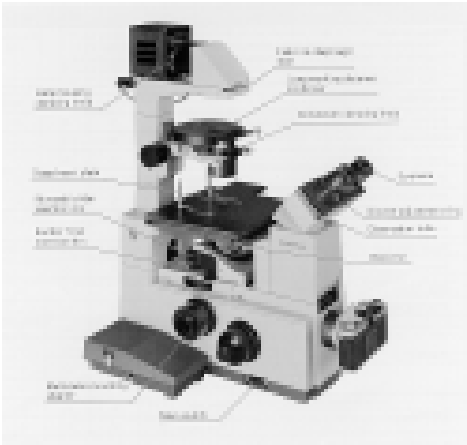

Figure 1.10 Olympus CK12 inverted microscope

Materials

- Inverted phase microscope
- Monolayer cultured cells or a suspension of protozoa in a plastic disposable petri plate

Procedure

1. Using Figure 1.10 as a guide, familiarize yourself with the components of the inverted microscope. Note that the operation of the microscope is the same as in Exercise 1.1, the only exception being that the components are inverted. The phase condenser may be an annular ring or a phase slider as depicted in Figure 1.10.
2. Place either a culture flask containing a monolayer of cells or a plastic petri plate 16 containing a

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suspension of protozoa on the stage of the microscope.

3. Observe the culture with normal bright field and with phase contrast. Note any movements of the cells and over a period of time, note any changes in the nuclear material of the monolayer cells.
4. Draw and label your observations in the space provided.

Exercise 1.11 - The Transmission Electron Microscope

LEVEL III

Figure 1.11 Zeiss EM9-S transmission electron microscope

Materials

- Transmission electron microscope facility

Procedure

1. Visit an electron microscope facility.
2. Familiarize yourself with the basic operations of Fixation, embedding and sectioning of materials for viewing in an electron microscope.

   If permitted, identify the primary parts of the TEM, including is various controls and operation. Refer to Figure 1.11 for details of the Zeiss EM9-S microscope.

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3. Complete the following operations.
    - Insert and Remove a Specimen from the chamber.
    - Turn on the high voltage, check current levels and turn on the beam.
    - Find and center the beam.
    - Use magnification controls and condenser.
    - Use binocular viewing screen
    - Take an electron photomicrograph.

Exercise 1.12 - Comparison of Electron Micrographs

LEVEL III

Materials

Procedure

1. Compare the transmission electron microscope image of the cell organelles in Figure 1.12 to the image of that organelle in the light microscope (Exercise 1.7).
2. List the organelles observed with each type of microscope, indicate their size and diagram a comparison of each organelle observed at the two levels.

Endnotes

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1. For absolute maximum resolution, oil should be used between the condenser and the slide as well, although in practice this is rarely done.
2. Blue has an additional advantage in that the eye perceives light blue as white . that is the reason for adding bluing to white laundry, to make it look brighter. green light, is nearly as good for resolution as blue and is more pleasing to the eyes. we can withstand longer and more intense observations with green light (note the color of computer monitors and most automobile dash lights). if a green filter is available, replace the blue filter with the green -- be sure to remove the blue -- and note any differences.
3. Since the electrons are shot down the column, the electron source is known as the electron gun, and the electrons are drawn off of the tungsten wire by a low voltage gun cap, then accelerated by the high voltage anode.
4. It should be noted that for most routine cell biology, a resolution of about 30 Å is all that is required. Electron microscopes will give resolutions of around 5-7 Å, and the top research microscopes are beginning to approach the theoretical limit of just above 1 Å. as we approach this theoretical limit, however, we enter the can't see the forest for the trees syndrome, or in more perspective, can't see the forest for the chlorophyll molecules syndrome. The cost of the instrument increases exponentially with increased resolution and extremely high resolution electron microscopes are used for research on electron optic theory, rather than for biology. The one advantage of high voltage EM for biologists is the ability to view thicker specimens, even hydrated living cells.
5. The directions are given for the Nikon Model S-Cb, but will apply to most microscopes, provided allowances are made for variations in manufacturer design.

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6. Exceptions to this occur when you are not attempting to use maximum resolution and are willing to trade resolution for some other purpose, such as ease of operation, or to compensate for the lack of a rheostat.
7. The Nikon S-Cb is provided with coaxial, coarse and fine focus knobs, both of which are located near the base. Clockwise rotation of either of the focus knobs by the operator lowers the microscope stage. other microscopes may have two separate focus knobs, and may alter the position of the upper lens tubes and/or the objectives rather than the stage.
8. On the Nikon S-Cb models, the most confusing and potentially damaging part of the microscope is the tension adjustment device for the coarse adjustment, and the preset device. incorrect adjustment of these will result in certain damage to the focus adjustment of the microscope, necessitating complicated repair.
9. For the Nikon microscopes equipped with a pre-set device, the lever should remain in its counter-clockwise position -- be sure to check this position periodically during the use of the microscope. this device has proven to cause enough problems that many laboratories are resorting to removing the lever from the microscope, to prevent damage to the focusing gears caused by improper use.
10. Type A immersion has a lower viscosity than Type B and is much preferred. Some laboratories will mix A and B to obtain intermediate viscosities. In any case, the oil should be a non-drying and with low fluorescence.
11. If the image observed with the 40X appears dull and with a milky halo to it, the first thing to suspect is oil on either the front of the lens or the slide. the second thing to check is the presence of oils and/or make-up from the eye lids on the oculars.
12. The average is statistically more valid, since there may be differing concentrations within various areas of the slide. A blood smear will definitely have variations since it will be normally a gradient from one end of the slide to the other, due to the means of preparing the smear.
13. If desired, whole blood may be substituted, diluted 1:200 with saline prior to use.
14. The coverslip used for the hemacytometer is especially thick, in order to prevent sagging in the middle. A regular microscope coverslip should never be used as a substitute, as it will yield incorrect volumes, often varying with each count.
15. Most bright field microscopes come with or can be easily fitted with a dark field stop. If your microscope does not have the disk supplied, try taping an ordinary dime into the filter holder below the condenser, and moving the condenser to a position where light can move in a ring around the coin. For many microscopes, this is an economical method of producing a dark field microscope. Alternatively, copy the image of a darkfield disk, size to fit your filter holder and cut it out of cardboard, construction paper or aluminum foil. Place it or tape it in the filter holder, or directly onto the condenser. The important feature is to keep the light moving in a cone around the periphery of the lens, and not through the center of the lens.
16. Plastic plates are preferred for this purpose, since the plastic has a more uniform thickness than ordinary glass petri plates. Special glass plates are available with ground optically flat glass in the bottom, but these are both expensive and fragile.

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Chapter 2: Histochemistry

Introduction

A few cell types are thin enough to be viewed directly in a microscope (algae, protozoa, blood, tissue cultures), but most tissues (kidney, liver, brain) are too thick to allow light to be transmitted through them. The tissues can be sliced into very thin sections provided they are first processed to prevent cell damage. The processing involves a series of steps; **fixation, dehydration, embedment** and subsequent sectioning with a **microtome**. These steps, explained in detail later in this chapter, are time consuming and often alter the cell structure in subtle ways. Fixing cells with formaldehyde, for example, will preserve the general organelle structure of the cell, but may destroy enzymes and antigens which are located in the cell.

Pathologists routinely examine tissues which have been fixed in formaldehyde and embedded in paraffin wax prior to sectioning. The process requires a minimum of 24 hours, and usually more if automated equipment is not available. This time delay can be crucial when a diagnosis of benign or malignant cancer is at stake. Valuable time can be saved by skipping the fixation and dehydration steps required for paraffin embedding, and freezing the tissue in a modified microtome, the **cryostat**. Sections can be prepared within minutes and diagnoses made while the patient remains on the operating table. Additionally, frozen sections will more often retain their enzyme and antigen functions. The use of frozen sections can reduce the processing time, but it is not a panacea. Freezing is not adequate for long term preservation of the tissues and the formation of ice crystals within the cells destroys subcellular detail. Frozen sections are also thicker since ice does not section as thin as paraffin. This results in poor microscopic resolution and poor images of what subcellular structures remain. If time or enzyme function is critical frozen sections are the preferred process. If subcellular detail is important, other procedures must be used. Selection of the correct procedure depends on what the cell biologist is looking for and to a point, becomes an art form. The histologist must choose among hundreds of procedures to prepare tissues in a manner that is most appropriate to the task at hand.

Fixation

Since cellular decomposition begins immediately after the death of an organism, biologists must fix the cells to prevent alterations in their structure through decomposition. Routine fixation involves the chemical cross-linking of proteins (to prevent enzyme action and digestion) and the removal of water to further denature the proteins of the cell. Heavy metals may also be used for their denaturing effect.

A typical laboratory procedure involves the use of an aldehyde as the primary fixative. Glutaraldehyde is used for transmission electron microscopy (TEM), and formaldehyde is used for routine light microscopy. The formaldehyde solution most often employed was originally formulated by Baker in 1944.

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Baker's Formalin Fixative contains:

calcium chloride	1.0 g
cadmium chloride	1.0 g
formalin, concentrated	10.0 ml
distilled water	100.0 ml

Blocks of tissue (liver, kidney, pancreas, etc.) of approximately 1 cm^3 are rapidly removed from a freshly killed organism and placed in the fixative. They are allowed to remain in the fixative for a minimum of four hours but usually overnight. The longer the blocks remain in the fixative, the deeper the fixative penetrates into the block and the more protein cross--linking occurs. The fixative is therefore termed progressive. Blocks may remain in this fixative indefinitely, although the tissues will become increasingly brittle with long exposures and will be more difficult to section. While it is not recommended, sections have been cut from blocks left for years in formalin.

Formalin has lately been implicated as a causative agent for strong allergy reactions (contact dermatitis with prolonged exposure) and may be a carcinogen ---- it should be used with care and always in a well ventilated environment. Formalin is a 39% solution of formaldehyde gas. The fixative is generally used as a 10% formalin or the equivalent 4% formaldehyde solution. The key operative term here is gas -- formaldehyde should be handled in a hood, if possible. As a gas, it is quite capable of fixing nasal passages, lungs and corneas.

Dehydration

Fixatives, such as formaldehyde, have the potential to further react with any staining procedure which may be used later in the process. Consequently, any remaining fixative is washed out by placing the blocks in running water overnight or by successive changes of water and/or a buffer. There are myriad means of washing the tissues (using temperature, pH and osmotically controlled buffers), but usually simple washing in tap water is sufficient.

If the tissues are to be embedded in paraffin or plastic, all traces of water must be removed: water and paraffin are immiscible. The removal of water is **dehydration**. The dehydration process is accomplished by passing the tissue through a series of increasing alcohol concentrations. The blocks of tissue are transferred sequentially to 30%, 50%, 70%, 80%, 90%, 95%, and 100% alcohols for about two hours each. The blocks are then placed in a second 100% ethanol solution to ensure that all water is removed. Note that ethanol is hydroscopic and absorbs water vapor from the air. Absolute ethanol is only absolute if steps are taken to ensure that no water has been absorbed.

It is important to distinguish between dehydration and drying. Tissues should **NEVER** be allowed to air dry. Dehydration involves slow substitution of the water in the tissue with an organic solvent. For comparative purposes, consider the grape. A properly dehydrated grape would still look like a grape. A dried grape is a raisin. It is virtually impossible to make a raisin look like a grape again, and it is equally impossible to make a cell look normal after you allow it to dry.

Embedding

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After dehydration, the tissues can be embedded in paraffin, nitrocellulose or various formulations of plastics. Paraffin is the least expensive and therefore the most commonly used material. More recently, plastics have come into increased use, primarily because they allow thinner sections (about 1.5 microns compared to 5--7 microns for paraffin).

Paraffin

For paraffin embedding, first clear the tissues. Clearing refers to the use of an intermediate fluid that is miscible with ethanol and paraffin, since these two compounds are immiscible. Benzene, chloroform, toluene or xylol are the most commonly used clearing agents, although some histologists prefer mixtures of various oils (cedarwood oil, methyl salicylate, creosote, clove oil, amyl acetate or Cellosolve). Dioxane is frequently used and has the advantage of short preparation times. It has the distinct disadvantage of inducing liver and kidney damage to the user and should only be used with adequate ventilation and protection.

Be wary of all organic solvents. Most are implicated as carcinogenic agents. Heed all precautions for the proper use of these compounds.

The most often used clearing agent is toluene. It is used by moving the blocks into a 50:50 mixture of absolute ethanol:toluene for two hours. The blocks are then placed into pure toluene and then into a mixture of toluene and paraffin (also 50:50). They are then placed in an oven at 56 - 58° C (the melting temperature of paraffin).

The blocks are transferred to pure paraffin in the oven for 1 hour and then into a second pot of melted paraffin for an additional 2--3 hours. During this time the tissue block is completely infiltrated with melted paraffin.

Subsequent to infiltration, the tissue is placed into an embedding mold and melted paraffin is poured into the mold to form a block. The blocks are allowed to cool and are then ready for sectioning.

Plastic

More recent developments in the formulation of plastic resins have begun to alter the way sections are embedded. For electron microscopy that requires ultrathin sections, paraffin is simply not suitable. Paraffin and nitrocellulose are too soft to yield thin enough sections.

Instead, special formulations of hard plastics are used, and the basic process is similar to that for paraffin. The alterations involve placing a dehydrated tissue sample of about 1 mm^3 into a liquid plastic which is then polymerized to form a hard block. The plastic block is trimmed and sectioned with an ultramicrotome to obtain sections of a few hundred Angstroms. Table 2.1 presents a comparison of paraffin embedding with the typical Epon embedment for TEM.

Softer plastics are also being used for routine light microscopy. The average thickness of a paraffin-sectioned tissue is between 7 and 10 microns. Often this will consist of two cell layers and, consequently lack definition for cytoplasmic structures. With a plastic such as Polysciences JB--4 it is possible to section tissues in the 1--3 micron range with increased sharpness. This is particularly

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helpful if photomicrographs are to be taken. With the decrease in section thickness, however, comes a loss of contrast, and thin sections (1 micron) usually require the use of a phase contrast microscope as well as special staining procedures. The sharp image makes the effort worthwhile.

Table	Light	Electron
Sample Size	1 cm³	1 mm³
Fixative	Formaldehyde	Glutaradehyde
Post-Fixation	None	Osmium Tetroxide
Dehydration	Graded Alcohol	Alcohol or Acetone
Clearing Agent	Xylol/Toluene	Propylene Oxide
Embedding Material	Pfaffin	Various Plastics
Section Thickness	5-10µ	60-90 nm
Stains	Colored dyes	Heavy Metals

Table 2.1 Light and electron microscopy preparations.

These soft plastics can be sectioned with a standard steel microtome blade and do not require glass or diamond knives, as with the harder plastics used for EM work.

Sectioning

Figure 2.1 AO microtome for paraffin sectioning

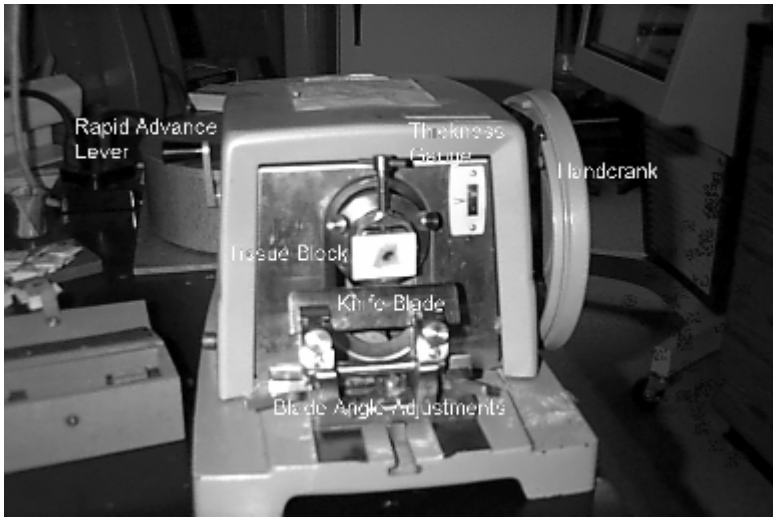

Figure 2.1 presents a photograph of the AO standard microtome. This device is found universally in cell biology laboratories and remains a fundamental instrument for histology.

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This rather simple device consists of a stationary knife holder/blade and a specimen holder which advances by pre-set intervals with each rotation of the flywheel mounted on the right hand side. In operation, it is similar to the meat and cheese slicers found within delicatessans. A control knob adjusts internal cams which advance the paraffin block with each stroke. It is relatively easy to section paraffin at 10 microns but requires a lot of skill and practice to cut at 5 microns. Since each section comes off of the block serially, it is possible to align all of the sections on a microscope slide and produce a serial section from one end of a tissue to the other.

While virtually anyone can cut a section within minutes of being introduced to the microtome, proper use of the microtome is an art form and requires practice and inventiveness. Many a cell biology research project has depended on the skills inherent in the use of this instrument. A microscope is nearly useless without a good thin, flat, and undistorted section from properly fixed, dehydrated and embedded tissue.

The Ultramicrotome

Figure 2.2 Sorvall ultramicrome

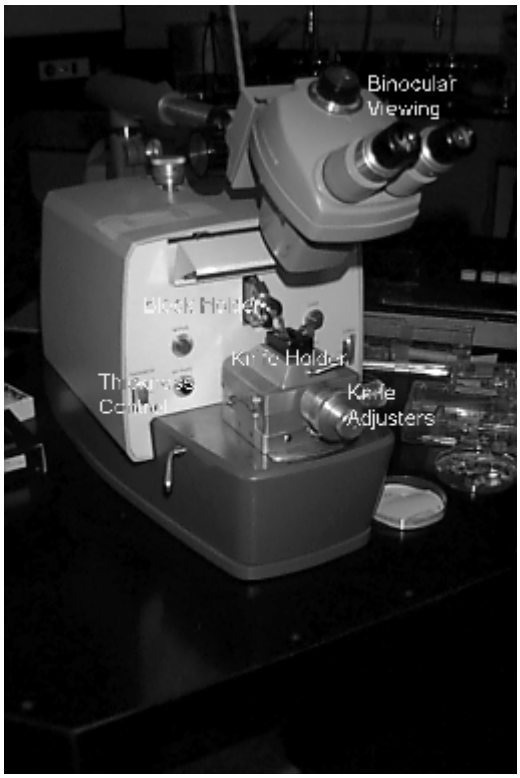

Figure 2.2 presents a view of an ultramicrotome. In principle, it is the offspring of the standard microtome, in that it also is a mechanical device that involves a stationary knife (glass or diamond) and a moving specimen. The specimen, or block, is a plastic embedded tissue that advances in nanometers rather than microns.

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Operationally, the only difference is that smaller samples are handled, which in turn requires a binocular dissecting microscope mounted over the blade. The tissue sections are too thin to see their thickness with the naked eye, one usually estimates thickness by the color of the diffraction pattern on the section as it floats off the knife onto the surface of a water bath. The sections are also too thin to be handled directly, and they are therefore transferred with wire loops, or picked off the water directly onto an EM grid.This process requires a good light source mounted to cast the light at just the correct angle to see the color pattern.

Since the plastics are hard enough to break steel knives, freshly prepared glass knives or commercially available diamond knives are used. A glass knife costs several dollars each, while a good diamond knife will cost in excess of $3,000. Either can be permanently damaged with a single careless stroke by the operator. Diamond knives are used in research laboratories by trained technicians because they have the advantage of a consistent knife edge (unlike glass which varies with each use) and can last for years if treated properly. They can usually be resharpened several times before discarding.

To minimize vibrations (which lead to uneven sections) ultramicrotomes are cast in heavy metal, are mounted on shock absorbent tables and, preferrably, kept in draft free environments of relatively constant temperature. To further minimize vibrations, some manufacturers have replaced the block's mechanical advance mechanism with a thermal bar, which advances the tissue by heating a metal rod. This can be exquisitely precise and is the ultimate in thin sectioning. Of course with this advancement comes increased cost and maintenance, and decreased ability to withstand rough treatment. The mechanically advanced ultramicrotome remains as the workhorse of the cell biology laboratory.

The Cryostat

Figure 2.3 Modern cryostat

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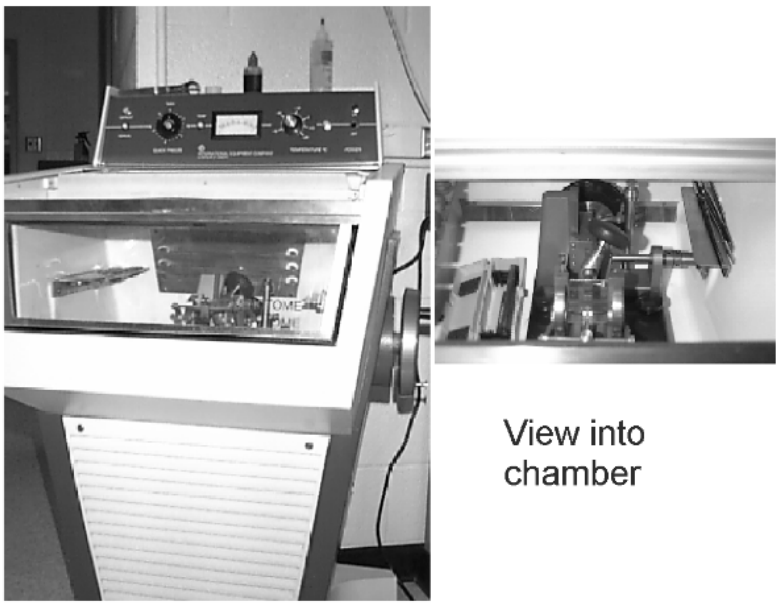

External View

Whether the sectioning is performed with a microtome or an ultramicrotome, one of the major delays in preparing a tissue section is the time required to dehydrate and embed the tissue. This can be overcome by direct sectioning of a frozen tissue. Typically a piece of tissue can be quick frozen to about -15 to -20 °C (for light microscopic work) and sectioned immediately in a device termed a cryostat. The cryostat is merely a microtome mounted within a freezer box. Figure 2.3 presents a modern cryostat.

A piece of tissue is removed from an organism, placed onto a metal stub and covered with a viscous embedding compound to keep it in a form convenient for sectioning. The stud and tissue are placed within the cryostat and quick frozen.

This method has the advantage of speed, maintenance of most enzyme and immunological functions (fixation is unnecessary) and relative ease of handling (far fewer steps to manipulate). It has the disadvantage that ice crystals formed during the freezing process will distort the image of the cell (bursting vacuoles and membranes for example) and the blocks tend to freeze-dry or sublimate. Thus, the blocks must be used immediately and great care must be taken to guard against induced artifact from the freezing process.

When temperature-sensitive (or lipid-soluble) molecules are to be studied, or where speed is of the essence (such as pathological examination during an operation) this is the preferred method. Sectioning operation with the cryostat is similar to that of the microtome, with the exception that one handles single frozen sections and thus all operations must be handled at reduced temperatures.

Exercise 2.1 - Selective Staining: Prepared Slides

LEVEL I

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Materials

- Prepared slides of:
  - DNA by Feulgen reaction
  - DNAase treated Feulgen reaction
  - RNA by methyl green -- pyronin
  - RNAase treated methyl green -- pyronin
  - PAS or Best carmine positive granules
  - Amylase treated PAS or Best carmine sections
  - Sudan black or osmium stained lipid
  - Lipase treated sudan black sections
- Microscope

Procedure

1. Obtain one of the prepared slides which has not been treated with an enzyme. Examine the slide carefully and draw a representative field of view.
2. Select a corresponding enzyme treated section and draw a representative field. Note any structures present in the untreated slide which are absent in the enzyme treated slide.

Notes

Each preparation in this exercise has a control (untreated) and enzyme treated slide. Each pair should be examined and compared. The pairs represent a view of similar cells which have had something removed by enzymatic digestion. You should determine which structures were removed by the enzyme treatment. Figure 2.4a presents an image of feulgen stained liver cells (left) and a similar section (right) stained identically, but pretreated with the enzyme DNAase. There are prominent nuclei present before DNAase treatment, but they are absent after treatment with the enzyme. Thus, the conclusion that nuclei contain significant amounts of DNA.

Exercise 2.2 - Basophilia

LEVEL I

Materials

- Paraffin sections of Carnoy fixed tissue (liver, kidney)
- Graded series of ethanol, 50, 70, 80, 95 and 100% (v/v)
- Mayer hematoxylin
- Scott solution
- Eosin
- Toluidine blue
- Xylol
- Coverslips and Permount
- Microscope

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Procedure

1. Obtain a slide from the instructor. Previously cut paraffin sections have been mounted on slides, and deparaffinized with xylol. They will be brought to the lab in Coplin jars filled with xylol. You will remove the slides from the xylol and transfer them to the indicated solutions which follow:

   Do not let the slides dry at any point in the procedure

2. Transfer the slides sequentially to Coplin jars containing (leave in each solution for the time indicated in parentheses):
   - Absolute Ethanol (1 minute)
   - 95% ethanol (1 minute)
   - 80% ethanol (1 minute)
   - 70% ethanol (1 minute)
   - 50% ethanol (1 minute)
   - Distilled water (1 minute)
   - Mayer hematoxylin (3 minutes)
   - Running water (3 minutes)
   - Scott solution (3 minutes)
   - Eosin (30 seconds)
   - Dip 2X in 95% ethanol
   - Dip 2X in Absolute ethanol
   - Dip 5X in tap water
   - Stain in toluidine blue (10 seconds)
   - Dip rapidly in distilled water
   - Dip once in 95% ethanol.
   - Dip 5X each in two changes of absolute ethanol
   - Place in Absolute ethanol (20 seconds)
   - Place in Xylol (2 minutes)
   - Place in Second Xylol (2 minutes)
3. Remove the slides from xylol, add a drop of permount and a cover slip.
4. Examine the slides with the low (10X) and high dry (40X)objectives of a light microscope. Do not use the oil immersion lens -- the permount will take several days to dry sufficiently and the slides should be treated carefully. If you wish to keep your slides, ask your instructor how to dry them.

   Be particularly careful not to get permount on the lenses.

Figure 2.4b Toluidine blue stained liver (basophilia)

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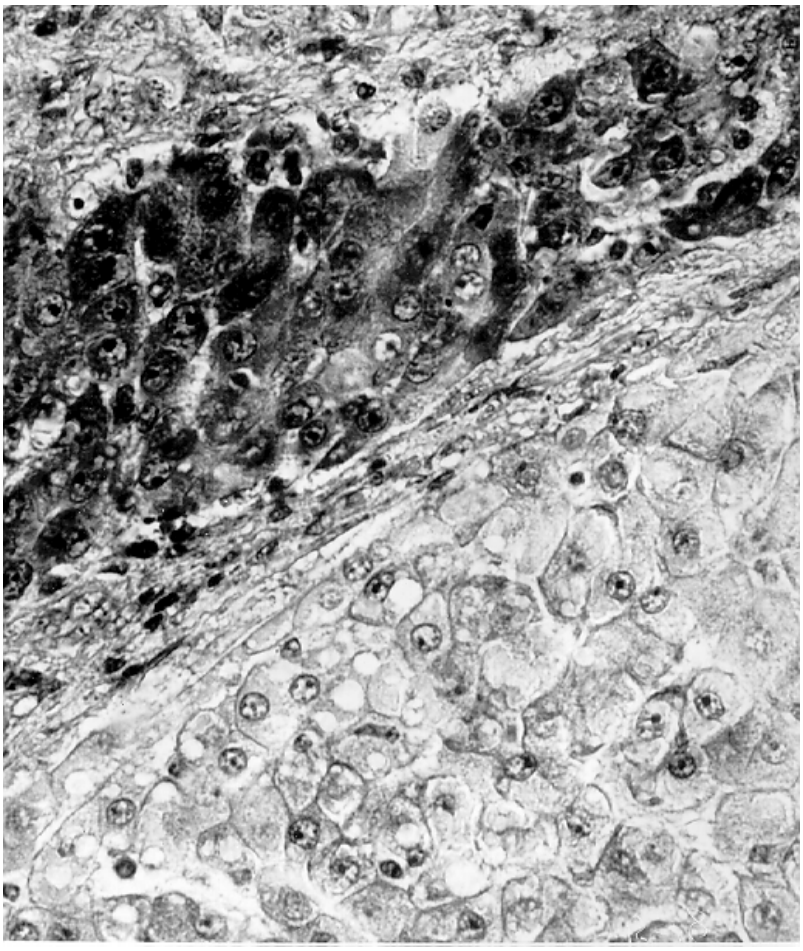

5. Draw each slide and compare to the commercial preparations of basophilic stained material.

Notes

This technique was originally designed for the analysis of Mast Cells to demonstrate the differential staining of cells. It will result in cells with pink cytoplasm and blue- -purple nuclei. This is the standard color given to animal cells, just as plant cells are usually dyed green with red nuclei. Of course, the choice of color is purely a matter of personal preference.

With the basophilic stain used here, collagen (an extracellular material) will stain pink, and erythrocytes (red blood cells) will stain red. The procedure is a modification of the standard H&E stain (hematoxylin/eosin) used by most cytologists. The procedures adds the use of toluidine blue, a metachromatic dye. That is, the dye has different colors depending upon the chemical nature of its polymerization. Toluidine blue is a basophilic dye, implying that it stains acid compounds (such as DNA and RNA and acid mucopolysaccharides). Staining with basophilic dyes such as toluidine blue (or methylene blue) is known as basophilia. Intensive staining with these dyes is known as hyperbasophilia and is often used in clinical pathological diagnoses.

Exercise 2.3 - Periodic Acid Schiff (PAS) Reaction

LEVEL I

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Materials

- Carnoy fixed paraffin sections 1
- Ethanol series, 50, 70, 95 and 100% (v/v)
- 1% Amylase in 0.1 M sodium phosphate buffer, pH 7.0
- 0.1 M Sodium phosphate Buffer, pH 7.0
- Incubator at 37° C
- Periodic acid
- Schiff's Reagent
- Sulfurous acid
- Xylol, Permount and coverslips
- Microscope

Procedure

1. Deparaffinize the sections by passing them through two changes of xylol (2 minutes each).
2. Rehydrate the sections by passing them through 100%, 95%, 70% and 50% alcohols (1 minute each).
3. Rinse gently in distilled water (2 minutes)
4. Pass the slides sequentilly through the following:
    - Place a slide in 1% buffered amylase at 37° C (1 hour). Place another slide in buffer at 37° C (1 hour)
    - Oxidize both slides in periodate (5 minutes)
    - Rinse gently in fresh distilled water (1 minute)
    - Stain in Schiff's reagent (15--30 minutes)
    - Bleach in sulfurous acid to remove any non--specific stain (15-30 minutes)
    - Rinse gently in fresh distilled water (5 minutes)
    - 70% alcohol (5 minutes)
    - 95% alcohol (5 minutes)
    - 100% alcohol (5 minutes)
    - Xylol (5 minutes)
5. Mount in Permount
6. Compare the amylase treated slides with those not so treated. Amylase removes amylose, animal starch. Those structures present without enzyme treatment, but missing after treatment are composed of amylose. Remember that the staining reagent is for aldehydes. Any compound with an aldehyde will turn pink with this reaction. Careful controls must always be used and even greater care taken in the final analysis of stained structures.

Notes

The major carbohydrates which remain in cells affixed to slides are the rather insoluble glucose polymers: glycogen, starch, and cellulose. All three polysaccharides give a positive periodic acid--Schiff (PAS) reaction. This staining procedure can be used on a variety of plant and animal material to determine the presence and intracellular localization of these polysacchrides.

Periodic acid is an oxidizing agent which breaks the C- -C bond between two adjacent hydroxyl groups. The 1,2--diol group in glucose is converted into a dialdehyde and any carbonyl groups are converted to carboxilic acids. The advantage of periodic acid lies in the specificity of its oxidation.

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It forms aldehydes within the polysacchride molecule but it does not continue the oxidation of the polymers to low molecular weight water soluble forms. Thus, glycogen, starch, and cellulose contain dialdehyde groups after the periodate treatment and are left in the cell in insoluble forms which can then be treated with Schiff's aldehyde reagent to form a purple colored product.

Exercise 2.4 - Methyl Green-Pyronin Staining of DNA

LEVEL I

Materials

- Cryostat
- Cold subbed slides
- Acid alcohol
- n-butanol
- 1 N HCl
- Methyl green
- Pyronin Y in acetone
- Incubator at 37° C
- Oven at 60° C
- 0.1% (w/v) RNAase in 0.1 M sodium phosphate buffer, pH 7.0
- 0.1 M Sodium phosphate buffer, pH 7.0
- Xylol, Permount and coverslips
- Microscope

Procedure

1. Remove a sample of tissue from a freshly sacrificed animal and freeze immediately for use in a cryostat.
2. Cut 7-10 sections and immediately fix in acid--alcohol (2 min.)
3. Rinse the fixed sections gently in distilled water (2 min.) and pass the sections through the following:
    - Digest one slide in buffered 0.2% RNase at 37° C (1 hour)
    - Digest second slide in 1N HCl at 60° C (1 hour)
    - Digest third slide in buffer only at 37° C (1 hour)
    - Rinse all slides gently in fresh distilled water
    - Stain in 0.2% methyl green (4 min.)
    - Blot excess methyl green from slides
    - Rinse in n-butanol (5 min)
    - Stain in 0.6% pyronin Y in acetone (1 min.)
    - Xylol (5 min.)
4. Mount in Permount
5. Observe and draw the tissues in the space provided. Compare the cells treated with RNAase to those treated with HCl and buffer only.

Notes

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The methyl green-pyronin procedure uses the high net negative charge of nucleic acids. Methyl green is a cation which binds rather specifically to DNA and thus serves as a convenient means of staining nuclei in both f ixed material and living cells. Pyronin, a red dye, is fairly specific for RNA with some binding to protein.

Control slides are important in interpreting the results of methyl green-pyronin staining, since the procedure is readily susceptible to artifact. One or both of the nucleic acids should be removed either enzymatically or by acid extraction.

Exercise 2.5 - The Feulgen Procedure for DNA

LEVEL I

Materials

- Carnoy fixed, paraffin sections of tissue 2
- Oven at 60° C
- 1 N HCl
- Schiff Reagent
- Sulphurous acid
- Graded ethanol series, 50, 70, 95 and 100% (v/v)
- Xylol, Permount and coverslips
- Microscope

Procedure

1. Deparaffinize slides by passing through two changes of xylol. Rehydrate the slides by passing through 100%, 95%, 70% and 50% alcohols.
2. Rinse gently in distilled water (2 min.)
3. Pass the slides sequentially through the following:
    - Extract one slide in 1N HCl at 60° C (12 min.)
    - Extract second slide in 1N HCl at 60° C (1 hour)
    - Extract third slide in water at 60° C (1 hour)
    - Rinse gently in fresh distilled water (1 min.)
    - Stain in Schiff's reagent (30 min.)
    - Bleach with sulphurous acid (10 min.)
    - Rinse gently in fresh distilled water (1 min.)
    - 70% alcohol (5 min.)
    - 100% alcohol (5 min.)
    - Xylol (5 min.)
4. Mount in Permount.
5. Draw representative cells from each slide in the box provided. If Figure 2.4 has been completed, compare the cells stained with methyl green-pyronin to those stained with the feulgen reaction. Be sure to include a comparison of any enzyme or acid hydrolyzed controls.

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The Feulgen procedure is undoubtedly the most widely used and most quantitative of all the cytochemical methods. Schiff's aldehyde reagent is the stain used. The great value of the Feulgen procedure is the manner in which cells are pretreated so that DNA furnishes the only available aldehyde to react with Schiff's reagent.

Extraction of cells with 1 N HCl at 60° C for 12 minutes provides optimum hydrolysis of purines from DNA, exposing the C1--aldehydes of deoxyribose.

The feulgen reaction is a semi--quantitative technique. If the only aldehydes remaining in the cell are those produced from the hydrolysis of DNA, then the technique is quantitative for DNA. It is possible to use an instrument known as a microdensitometer or microscpectrophotometer to actually measure the intensity of the pink feulgen reaction for a given organelle. Using this procedure, it was early determined that interphase cells were composed of two populations, those with 2C level of DNA and those with 4C. The nuclei looked identical, but one contained twice as much DNA within it.

This gave rise to the division of the interphase period of the cell cycle to a G1, an S, and a G2 based on the synthesis of that extra DNA.

For now, see if you can visually discern differences in the intensity of the feulgen stain reaction within the nuclei.

Exercise 2.6 - Alkaline Phosphatase Localization

LEVEL II

Materials

- Kidney sections (preferrably frozen sections)
- Cold acetone
- Chloroform
- Incubating Solution

0.8% (w/v) paranitrophenyl phosphate	2.0 ml
2% (w/v) sodium barbitol	2.0 ml
distilled water	1.0 ml
2% (w/v) calcium chloride	4.0 ml
5% (w/v) magnesium sulfate	0.2 ml

- Incubator at 37° C
- 2% (w/v) cobalt nitrate
- 2% (w/v) ammonium sulfide
- Acetone solutions 100, 80 and 50% (v/v) in water

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- Mayer's hematoxylin
- Scott solution
- Ethanol series, 50, 70, 95 and 100% (v/v)
- Xylol, Permount, slides, coverslips
- Microscope

Procedure

1. Use a cryostat to cut 10-15m frozen sections of kidney at -2° C. Mount on gelatin coated slides and dip in cold acetone to fix. Alternatively cut 10m paraffin sections of acetone or formaldehyde fixed materials. Acetone fixation will result in better enzyme preservation than formaldehyde. Tissues should be fixed in chilled acetone for 24 hours prior to paraffin embedding. Low temperature paraffin (560 melting temperature) works best and 10m sections need to be deparaffinized with chloroform before hydration through a series of acetones to water.
2. Incubate in incubating solution, 37° C for 30 minutes.
3. Wash in distilled water.
4. Place the slides in 2% cobalt nitrate for 5 minutes.
5. Rinse in distilled water.
6. Place the slides in 2% (w/v) yellow ammonium sulfide for 2 minutes. This step should be done in a ventilated hood to avoid the sulfide fumes.
7. Wash in distilled water.

   The slides can be viewed at this point with phase contrast or as a simple wet mount. If there is not sufficient black sulfide precipitate, the slides can be returned to the ammonium sulfide for an additional 2 minutes. If further contrast is needed, the cells can be counterstained with any basophilic stain. The following uses Mayer

   - Place in Mayer's hematoxylin (3 minutes)
   - Place in running water (3 minutes)
   - Place in Scott solution (3 minutes)
8. Dehydrate by passing through 50, 70, 95 and 100% ethanols, clear with xylol and mount a coverslip with permount.
9. Examine all slides and compare the distribution of alkaline phosphatase on the surface of the cells.

   Sites of alkaline phosphatase will be black, sharp and clear, nondiffuse. The reaction precipitates calcium phosphate as the phosphate is released by the enzyme. The calcium phosphate in turn is turned to black (or brown) prepicipate in the presence of the cobalt nitrate and ammonium sulfide.

Exercise 2.7 - Immunofluorescent Localization of Tubulin

LEVEL II

Materials

- Coverslip cultures of an appropriate monolayer cell line

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- Phosphate buffered saline (PBS)
- Acetone/Methanol (absolute) in a 50:50 volume mixture
- Rabbit anti-tubulin (or other primary antibody to tubulin)
- FITC-labeled goat anti-rabbit (or secondary antibody to match the primary)
- Fluorescence Mounting Media
- Fluorescent Microscope equipped with 490 nm excitation filter and 515 nmbarrier filter
- Kodachrome film or equivalent color slide film (Kodak Tri-X or Ilford HP400 may be substituted for black and white photography)

Procedure

1. Set up a coverslip culture of an appropriate cell line 24 hours prior to the lab. This is best accomplished by dry sterilization of #1 coverslips which are subsequently placed in plastic tissue culture plates. Cells are placed on the coverslips with sufficient media to cover and allowed to grow for 24 hours. There should be sufficient cells to view comfortably, but they should not be crowded on the slide.
2. Remove the coverslip from the culture plate and dip several times in a beaker of phosphate buffered saline (PBS) to rinse off the culture media. Drain, but do not allow to dry.
3. Immediately immerse in a 50:50 mixture of acetone/methanol at room temperature. Allow the coverslips to remain in the acetone/methanol for 2 minutes.
4. Remove the coverslips from the acetone mixture and rinse 2X with PBS.
5. Prepare a 1/40 anti-tubulin dilution using PBS. PbS alone may be used or better, augment the PBS with 3% (w/v) Bovine Serum Albumin (BSA).

   It may be necessary to check the appropriate antibody dilution. If so, make 1/10, 1/100, 1/1,000 and 1/10,000 dilutions to establish the correct titer. Working dilutions also may vary with the manufacturer - check the literature that accompanies your primary antibody.

6. Place the coverslip in a petri plate containing filter paper moistened with PBS. Make sure the cells are pointed up when placed in the petri plate! Flood the coverslip with 50 ml of 1/40 dilution of the primary antibody (or as determined in step 5).
7. Incubate at room temperature for 1-4 hours. The incubation may be left overnight if necessary.
8. Wash 3X with PBS. Place coverslips in a new petri plate containing PBS moistened filter paper.
9. Apply 50 ml of FITC-labeled second antibody. A 1/100 dilution usually is satisfactory. You may need to determine the appropriate dilution based on manufacturer directions or through trial and error dilutions in the range of 1/10 to 1/300.
10. Incubate for 30 minutes at room temperature.
11. Wash 3X with PBS.
12. Place a drop of glycerin or appropriate commercial fluorescent mounting media on a slide and place the coverslip onto the slide with the cells facing down into the glycerin.
13. Observe immediately with a fluorescent microscope adjusted for fluorescein (490 nm excitation and 515 barrier filter). The slides are best photographed using Kodak Ektachrome or equivalent with an ASA or 200-400. An exposure of 1-2 seconds is usually sufficient, although for low light, 30 seconds may be required. It is best to make a test exposure roll if a photometer is not available.

Figure 2.7 Microtubules stained by immunofluorescent labeling

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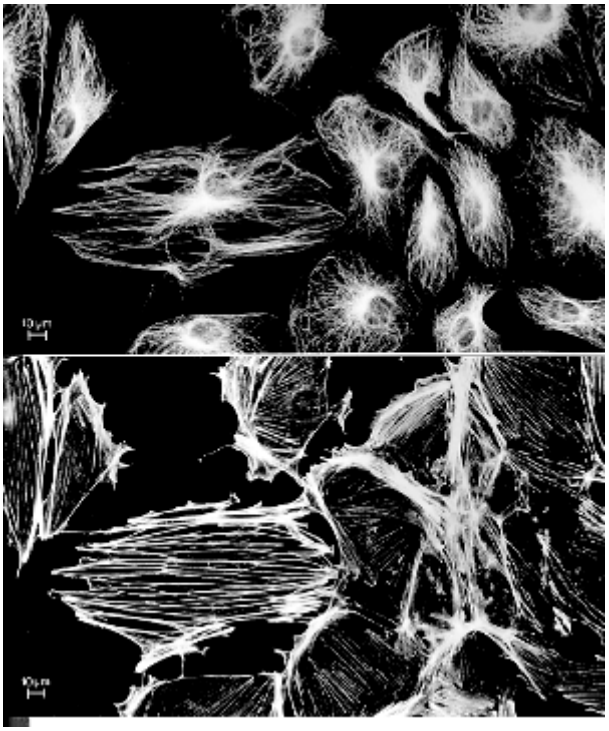

Image supplied courtesy of Carl Zeiss Oberkochen Technisch-Wissen-schaftliche Information by Dr. Wolfgang Pfeiffer.

Exercise 2.8 - Autoradiography

LEVEL III

Materials

- ³H-Thymidine, specific activity of 2.0
- curie/millimole
- Onion sets, jars and toothpicks
- Alcohol-acetic acid fixative
- Materials for feulgen reaction (Exercise 2.4)
- Paraffin embedding, sectioning equipment
- Kodak Nuclear Track Emulsion (or equivalent)
- Darkroom
- Water bath at 42° C
- Kodak D19 Developer
- Kodak Fixer
- Xylol, Permount and coverslips
- Microscope

Procedure

1. Carefully read and follow all precautions for the safe handling of radioactive materials (Appendix H).
2. Place toothpicks into the sides of an onion and set the onion into the top of a small jar or beaker. Fill the

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jar with water and allow the onion to begin root development (3- 7 days). Mild aeration of the water will assist in the growth of the roots.

3. Set up another beaker, containing a 10 µ /ml solution of ³H-thymidine such that there are approximately 10 µc/ml, transfer the onion and its growing roots to the jar containing the radioactive thymidine.
4. Allow the roots to stay in the radioactive solution for 1 hour at room temperature.
5. Remove the onion and hold the bulb over a beaker containing water. Rinse the onion roots by dipping several times in the beaker. Transfer the bulb to yet another beaker of aerated water and allow to remain in this beaker for four hours.
6. Cut off the roots tips and fix in alcohol-acetic acid (3:1) overnight. Change the fixative after the first four hours.
7. Wash the roots in water for several minutes and place in 1N HCl at 60° C for 12 minutes.
8. Stain the root tips with the Feulgen Reaction as given in Steps 3.b-3.e of Exercise 2.5.
9. Dehydrate the root tips, embed in paraffin and section at 10-15 microns. Mount on microscope slides.
10. Deparaffinize in xylol and rehydrate sections by passing first through a series of alcohols and finally in two changes of water.
11. In a dark room 3 melt some liquid autoradiographic emulsion at a water bath at 42° C.
12. Place two slides back-to-back and dip slowly into the melted emulsion. Remove, allow to drain and place in an appropriate light-proof container and allow them to dry in a vertical position.
13. When dry, place the slides into an opaque slide box containing drierite. Wrap in aluminum foil and place in a refrigerator.
14. The slides must stay in the refrigerator until a proper exposure has been made. This can vary from 5 days to over two weeks. After one week, a trial slide should be developed using Steps 15 and 16 below. Examine the slide at 10X with a bright field microscope and look for the presence of black silver grains located over the cells. A correct exposure is determined by the appearance of silver grains over the cells, but few or no silver grains located in areas without cells. If the appearance of the trial slide is correct, then the remainder should be processed immediately. Repeat an additional trial slide in 2-3 days, and repeat every three days thereafter until an adequate exposure is obtained.
15. Develop the autoradiogram emulsion in the darkroom as follows:
    - Develop in Kodak D-19 Developer at 20° C for 3 minutes.
    - Wash for 10 seconds in distilled water.
    - Fix with Kodak Fixer for 3 minutes.
    - Wash in running water for 15 minutes.
    - Dehydrate by placing in 95% alcohol for 3 minutes.
    - Place in 100% alcohol for 3 minutes.
    - Clear in two changes of xylol for 3 minutes each.
16. Mount coverslip with Permount.
17. Draw and label the autoradiograms in the space provided on the following page. Calculate the percent of cells actively undergoing DNA synthesis during the time of exposure to radioactive thymidine.

Note that the tissue and the silver grains are in different planes of focus and you will need to constantly switch focus from one plane to the other.

Notes

This procedure involves the incorporation of a radioactive substance into a cell, and subsequent detection of that material through the use of a photographic emulsion. The primary source used for cell biology is an organic molecule containing tritium, the radioactive form of hydrogen.

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Radioactive carbon, phosphorous and iodine are occasionally utilized, but tritium has inherently more resolution than any of the others.

Tritiated thymidine (³H-thymidine) is often used, for example, to study the synthesis and location of DNA. Thymidine is a soluble base which is specific to DNA. It is incorporated into the macromolecular structure of DNA during synthesis and replication of the chromosomes. Upon fixing the cells for standard histological examination, the DNA molecules (with their incorporated, radioactive, thymidine) are precipitated or cross-linked as permanent parts of the cell. Un-incorporated thymidine is removed from the cell, as it remains soluble and is disposed of in the tissue washing procedures.

When the tissues are sectioned and applied to a glass slide, they will contain radioactive nuclei, but only those nuclei that were in the S phase of division during the exposure of the cells to ³H-thymidine. Radioactive sources can not be detected directly, but if a photographic emulsion is applied directly over the section, it will become exposed by the radioactive source.

When the photographic emulsion is subsequently processed (i.e. developed), the exposed portions of the emulsion will contain reduced silver grains in direct proportion to the amount of radiation being given off beneath it (in the nuclei of our example).

If the exposed, developed slides are now examined with a microscope, there will be two layers of interest. Focusing the microscope on the tissue itself will give a view of the tissue and cell architecture. If the focus of the microscope is moved upward, however, the cells will go out of focus, exactly as the photographic emulsion comes into focus. Within the emulsion will be areas of reduced silver grains and clear areas containing no silver grains. By alternately focusing on the tissue and the emulsion, those nuclei that are radioactive can be readily identified.

If desired, the number of silver grains could be counted to give a quantitative measure of ³H-thymidine incorporation (and thus DNA synthesis), although this is a rather complex procedure to control, with any significant accuracy.

Endnotes

---

1. Sections may be cut directly from frozen blocks, if a cryostat is available. The sections should be fixed for 2 minutes in acid-alcohol, rehydrated and the procedure then begun with the amylase treatment (Step 4).
2. Frozen sections fixed in acid-alcohol may be substituted. Begin processing with distilled water wash (Step 2).
3. A Series 2 Wratten Filter with 15 watt bulb, which is at least 5 feet from the work area may be used, but reference should be made to the emulsion manufacturer's directions for safelight.

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Chapter 3: Cell Fractionation

Introduction

All of the procedures given in Chapter One have in common the use of a microscope. The basic principle for all microscopes is that the cell is composed of smaller physical units, the organelles. Definition of the organelles is possible with microscopy, but the function of individual organelles is often beyond the ability of observations through a microscope. We are able to increase our chemical knowledge of organelle function by isolating organelles into reasonably pure fractions.

A host of fractionation procedures are employed by cell biologists. Each organelle has characteristics (size, shape and density for example) which make it different from other organelles within the same cell. If the cell is broken open in a gentle manner, each of its organelles can be subsequently isolated. The process of breaking open cells is **homogenization** and the subsequent isolation of organelles is **fractionation**. Isolating the organelles requires the use of physical chemistry techniques, and those techniques can range from the use of simple sieves, gravity sedimentation or differential precipitation, to ultracentrifugation of fluorescent labeled organelles in computer generated density gradients.

Homogenization

Often, the first step in the preparation of isolated organelles is to obtain a "pure" sample for further analysis. Cells which are not attached to others (such as blood or suspension tissue cultures) can be separated if they have distinct shapes, densities or characteristics which can be marked (such as charge, antigen or enzyme presence). Cells which are part of a more solid tissue (such as liver or kidney) will first need to be separated from all connections with other cells. In some cases this can be performed by simply chelating the environment (removing Ca^{++} and/or Mg^{++}), but in most instances the cells will need to be enzymatically or mechanically disaggregated. This often results in subtle changes to the cells, and at a minimum will disrupt such cell-cell communications as DESMOSOMES and TIGHT JUNCTIONS.

Homogenization techniques can be divided into those brought about by osmotic alteration of the media which cells are found in, or those which require physical force to disrupt cell structure. The physical means encompass use of mortars and pestles, blenders, compression and/or expansion, or ultrasonification.

Osmotic alterations

Many organelles are easier to separate if the cells are slightly swollen. The inbibition of water into a cell will cause osmotic swelling of the cell and/or organelle, which can often assist in the rupture of the cell and subsequent organelle separation. The use of a hypo-osmotic buffer can be very

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beneficial, for example, in the isolation of mitochondria and in the isolation of mitotic chromosomes.

Mortars, Pestles

Perhaps the most common procedures use Ten Broeck or Dounce homogenizers, both of which are glass mortar and pestle arrangements with manufactured, controlled bore sizes. The addition of a motor driven teflon pestle creates the Potter-Elvijem homogenizer. Ultrasonification is a useful adjunct to this procedure, but is often sufficient by itself.

To obtain pure organelles, the cells must be ruptured, so that the cell membrane is broken, but the organelle to be studied is not. The process of rupturing a cell is known as **homogenization** of the cell. It also varies from simple mortar/pestle grinding (with the aid of sand or glass beads) for many plant materials, to repeated high velocity compression and expansion in what is known as a "French Press." The French Press is very powerful and can disrupt bacteria and viral particles as well. It is favored for use when **molecular dissociation** is required, such as in the separation of DNA from the nematode worm *C. elegans*. Often, cell rupture is aided by rapid freezing (in liquid nitrogen) and subsequent application of mechanical forces.

With all forms of homogenization, the shear force must be carefully controlled. Too little and the organelles will not be separated, too much and even the molecules can be broken.

Blenders

For molecular separations, mechanical blenders are often used, varying in sophistication from household blenders to high speed blenders with specially designed blades and chambers (e.g. a Virtis Tissue Homogenizer). The mechanical procedures are augmented by various organic solvents (for phase separations) and/or detergents to assist the denaturation and separation of molecules (e.g. DNA from histones). When specific molecules are sought, care must be taken to inhibit powerful degradation enzymes (such as RNase when extracting RNA). This can be accomplished by subjecting the specimen to cold temperature, or by adding specific organic inhibitors (Diethylpyrocarbonate for RNase), or both.

Compression/Expansion

For cellular material which is difficult to shear by the above mentioned techniques (plant cells and bacteria), a device known as a "French Press" is ocassionally used. This device forces a slurry of the cells through an orifice (opening) at very high pressures. The rapid expansion of the pressure from within literally "blows" the cells apart. While this technique is not often required, it is the only way to break open some materials. The units have capacities from 1 to 40 ml and can reach pressures of 20,000-40,000 pounds per square inch (psi).

Ultrasonification

Ultrasonicators have been used with increasing popularity to separate organelles from cells, particularly from tissue culture cells. Light use of an ultrasonic wave can readily remove cells from

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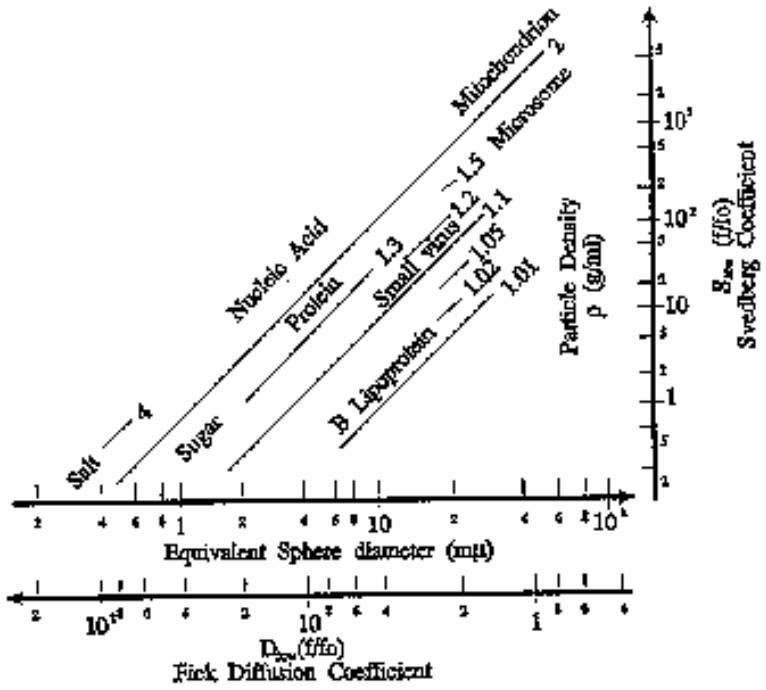
a tissue culture substrate (such as the culture flask). It can also be adjusted to merely separate cells, or to break open the plasma membrane and leave the internal organelles intact.

Figure 3.1 presents a few of the various devices used for homogenization.

Fractionation

Figure 3.2 Methods used for centrifugal separations



Gravity Sedimentation

Once the cells have been homogenized, the various components must be separated. For some materials (whole blood, cells in suspension), this can be accomplished by the simple use of gravity sedimentation. In this procedure, the samples are allowed to sit, and separation occurs due to the natural differences in size and shape (density) of the cells. Red blood cells are denser than white cells, and thus whole blood separates into an RBC-rich bottom layer, an intermediate "buffy coat" layer of WBC's and an upper plasma portion of settled blood samples (an anti-coagulant is added to prevent coagulation, which would interfer with the separation).

Centrifugation

Without question, however, the most widely used technique for fractionating cellular components is the use of centrifugal force. Procedures employing low speed instruments with greater volume capacity and refrigeration are known as "preparative" techniques. Analytical procedures, on the other hand, usually call for high speed with a corresponding lower volume capacity. A centrifuge working at speeds in excess of 20,000 RPM is an ultracentrifuge.

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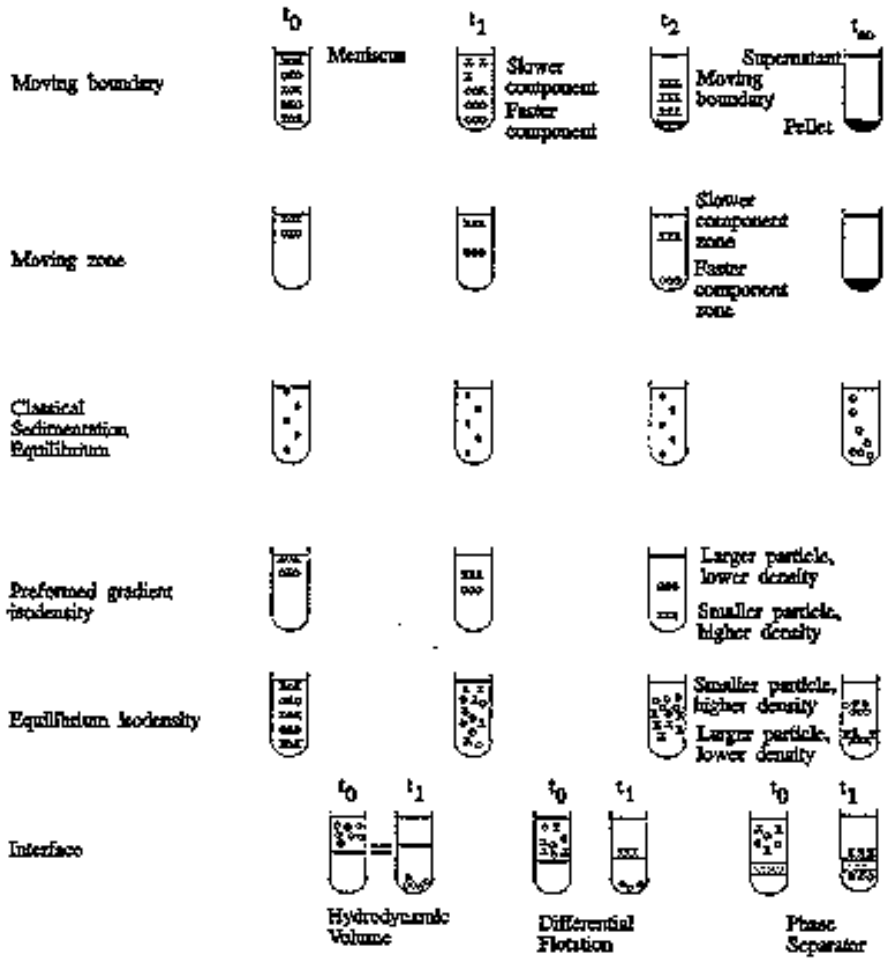
Organelles may be separated in a centrifuge according to a number of basic procedures. They can be part of a moving boundary, a moving zone, a classical sedimentation equilibrium, a preformed gradient isodensity, an equilibrium isodensity or separated at an interface. These are briefly diagrammed in Figure 3.2.

Physical Properties of Biological Materials

Figure 3.3 Physical properties of biological materials



Before undertaking the centrifugal separation of biological particles, let's discuss the particle behavior in a centrifugal force. Particles in suspension can be separated by either **sedimentation velocity**, or by **sedimentation equilibrium**. Sedimentation velocity is also known as **zone centrifugation** and has the advantage of low speed centrifugation and short times, but yields incomplete separations. Sedimentation equilibrium is also known as **isopycnic** or **density equilibration** and requires specimens to be subject to high speeds for prolonged periods of time. It has the advantage of separating particles completely.

Sedimentation Velocity

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Particles in solution will accelerate and attain a terminal velocity when subjected to a centrifugal force. This velocity is determined in part by the size, weight, density and shape of the particle, as well as the viscosity of the medium through which it must travel, and, of course, the centrifugal force generated. The terminal velocity is referred to as the sedimentation velocity of the particle and can bew used to measure the size, weight or density of the particle.

Sedimentation Coefficient

The sedimentation velocity (as terminal velocity) is measurable. The terminal velocity is dependent upon the relative centrifugal force (RCF) applied to the particle and is related to a mathematical factor, the **sedimentation coefficient** or **sedimentation constant**. This coefficient is given in Svedberg (S) units, so named for the Swedish pioneer of centrifugation theory and operation, T. Svedberg. The S units are measured as fractions of time, specifically 10^{-13} sec. The sedimentation coefficient is determined by dividing the terminal velocity by the centrifugal force field strength. The relationship of sedimentation coefficient to the diameter of a particle is visually presented in Figure 3.2.

Diffusion Coefficient

While a particle is reaching its terminal velocity, it is also effected by diffusion, usually in a direction opposite to its movement under force. This is a movement by Brownian motion and can be mathematically stated as a constant, the **diffusion coefficient** (D), which is the spread of the molecule divided by time. It is expressed in units, Ficks, which are 10^{-7} cm^2/sec. As noted in Figure 3.2, there is a direct relationship between the diffusion coefficient of a particle and its diameter. Both, in turn, are related to the sedimentation coefficient of the particle.

Both the sedimentation coefficient and the diffusion coefficient are corrected mathematically to express their values at 20° C, with water as the medium through which they move. They are almost never measured in water nor at this temperature, but formulas exist for the conversion to standard conditions.

What is important is that the S value can be measured and will give an important clue as to the physical structure and size of the particle. In practice, the S value is reasonably easy to determine.

The sedimentation coefficient is given by the formula:

$S = 1/\omega^2 r \times dr/dt$ (Equation 3.1)

where ω= angular velocity of the rotor in radians/sec calculated as 0.10472 x RPM

r = the distance between the particle and the center of rotation (mm)

dr/dt = the rate of movement of the particle (cm/sec)

The value of the sedimentation coefficient can be determined by timing the movement (velocity and distance) of a particle in a medium of known viscosity. The simplest means to do this is to centrifuge for a specific time with a known force, and to calculate the distance moved. Far more

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expensive, but quicker would be to monitor the movement of the particles while they actually are in the centrifugal field. This monitoring can be accomplished through the use of an "Analytical Ultracentrifuge" equipped with Schlierien Optics. Basically, this procedure takes stroboscopic photos of the advancing molecules within the centrifugal field. It is far too expensive an instrument to have in all but the best equipped molecular laboratories, however.

Sedimentation equilibrium

If a sample contains many different particles with differing densities and sizes, they will begin to separate on the basis of those parameters. The large particles will settle to the bottom of a tube faster than the smaller ones. If the relative centrifugal force is gradually increased, the time for the consequent separation of particles can be decreased.

By varying centrifugation force (speed) and time, while maintaining a continous media density, different sizes of particles can be separated on the basis of their size. Large particles, such as whole cells and nuclei are sedimented at low speeds. Mitochondria and chloroplasts require higher speeds and/or longer times of centrifugation. Ribosomes require even greater forces and longer times. Thus, it is possible to design a protocol which first sediments large organelles, and then by increasing the centrifugation time or speed to sediment smaller particles from the same tube. This protocol is known as differential centrifugation, and the process makes use of both time and speed. Since the procedure sediments large organelles first, they are often contaminated by the smaller organelles which start at the bottom of the centrifuge tube.

At the beginning, the pellet area (bottom of tube) will contain both small and large randomly distributed organelles. As the centrifuge is run, larger particles move down the tube, but smaller ones do not move up; the process is based on sedimentation, not flotation. As the process continues, the larger particles are removed and thus the smaller the particle, the purer the isolated fraction will be at later centrifugation steps. Of course, the smaller organelles are separated as both contaminants of the larger organelles, and as sediments of subsequent centrifugations. Thus, if you wish to maximize the collection of smaller organelles, or minimize the presence of smaller organelles in the large organelle fraction, it is necessary to recentrifuge the larger fractions several times and to collect and pool the resulting smaller units.

It is possible, however, to sediment and float the particles simultaneously. If the particles to be separated have differing densities (gm/ml) they can be separated through a medium that allows particles of one density to "float" and particles of higher density to sink to the bottom. Such media can be layered into the centrifuge tube in **step gradients** or **linear gradients**.

In either gradient, the particles are centrifuged until they reach a density equal to the media, thus the name **equilibrium density separations**. This process has been greatly utilized in the analysis of molecular weights for proteins and nucleic acids, since to a great extent, the density of these molecules can be directly related to their size. Equilibrium density is also used to successfuly isolate membranes and other high lipid-containing organelles.

Exercise 3.1 - Sucrose Fractionation of Castor Bean

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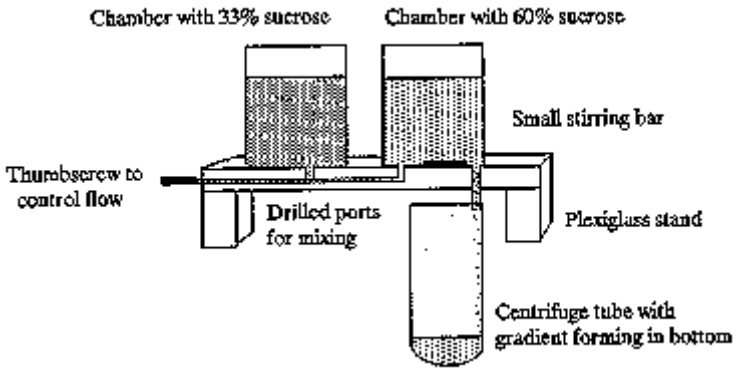
LEVEL I

Figure 3.4 Homemade gradient forming device



Materials

- Castor beans 1
- Gradient former Sucrose solutions in 0.01 M EDTA, pH 7.5

  33%, 44%, 50%, 57%, 60% (w/v) 2

- Grinding Medium
  - 0.4 M sucrose
  - 0.165 M Tris-HCl buffer, pH 7.5
  - 0.01 M KCl
  - 0.01 M $MgCl_2$
  - 0.01 M EDTA adjusted to pH 7.5
  - 0.01 M dithiothreitol
- Chopping board and knife or razor blade
- Mortar and pestle
- Cheesecloth
- Refrigerated centrifuge (ultracentrifuge preferred) with swinging bucket rotor
- Spectrophotometer and tubes
- Hemacytometer and phase contrast microscope

Procedure 3

1. Germinate castor beans by soaking overnight, followed by germination in moist vermiculite, or paper towels, at 30° C.

   Castor beans are poisonous and may be fatal if taken internally. Wear gloves while handling.

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2. After approximately 5 days, remove the embryos and cotyledons and discard. Wash the endosperm in distilled water and chill on ice.
3. Prepare two centrifuge tubes for sucrose gradients; one for a linear 33-60% gradient and the other for a stepped gradient. 4 The volume of each solution will depend on the centrifuge rotor to be used and its corresponding centrifuge tube capacity. The following are directions for 35 ml centrifuge tubes.

Stepped Gradient

Carefully layer, in order, the following solutions into a centrifuge tube

- 3 ml of 60% sucrose
- 6 ml of 57% sucrose
- 9 ml of 50% sucrose
- 9 ml of 44% sucrose
- 3 ml of 33% sucrose

Linear Gradient

Add 5 ml of 60% sucrose to the bottom of a centrifuge tube and form a linear 60 to 33% sucrose gradient on top of that. Using a gradient former, as shown in Figure 3.4, place 12 ml of 60% sucrose in the right chamber and 12 ml of 33% sucrose in the left chamber.

4. Combine 60 gm of endosperm tissue with 90 ml of grinding medium and chop vigorously.
5. Transfer the coarse material to a cold mortar and pestle and continue to grind until a fine paste is formed.
6. Filter the **BREI** (the paste from step 5) through two layers of cheesecloth. Collect the fluid into a beaker, transfer it to a centrifuge tube, and centrifuge the filtrate for 10 minutes at 270 xg to remove unbroken cells and large debris.
7. Decant the supernatant into a clean, cold centrifuge tube and recentrifuge for 30 minutes at 10,800 20xg. Resuspend the pellet in 5 ml. of grinding medium and hold on ice for further analysis.
8. Carefully decant the supernatant and save for subsequent analysis. Gently resuspend the pellet in 6 ml. of grinding medium.
9. Carefully layer 2 ml of the pellet onto a stepped sucrose gradient and 2 ml of the pellet onto a linear gradient.
10. Team up with another laboratory section and carefully balance your corresponding tubes. That is, be sure that the stepped gradients for both sections are exactly the same weight. Add grinding media to balance, where appropriate. Have the instructor check the balance before proceeding.
11. Centrifuge the tubes at 4° C for 4 hours at 25,000 RPM in a Beckman SW27 rotor or equivalent. 5
12. Upon completion of the centrifugation, fractionate the samples into approximately 20-30 equal portions. It is convenient to collect samples of 1.5 ml. This yields a volume suitable for subsequent spectrophotometric analysis without the use of small volume cuvettes.
    This is accomplished most rapidly by gently inserting a long 18 gauge needle, with the tip ground off, into the centrifuge tube so that it rests on the bottom of the tube. Without moving the needle (so as not to stir the contents), attach a 2.0 ml syringe and pull out the bottom 1.5 ml of the sample. Remove the syringe and place the 1.5 ml fraction into a test tube marked as #1. Re-attach the syringe without disturbing the gradient, and repeat as often as need to totally fractionate the gradient.

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Alternatively, the gradient can be fractionated by puncturing the bottom of the tubes with a needle, and collecting the fractions in 1.5 ml portions by counting the appropriate number of drops that drip from the punctured tube. There are several commercially available fraction devices designed for this purpose. If one is available, follow the manufacturer's directions.

13. Read the optical density (Absorbance) of each fraction in a spectrophotometer at 540 nm. Plot a double graph, with fraction number on the x axis (bottom to top) vs OD_{540} on the y axis, and fraction number vs % sucrose on the y axis. 6
14. Carefully examine all fractions with a phase contrast microscope. Identify and count (use a hemacytometer where appropriate) all structures found in each fraction.

Exercise 3.2 - Sedimentation Densities

LEVEL I

Materials

Table 3.1 Densities of cell structures in sucrose

Structure	Density (g/cm^3)
HGolgi apparatus	1.06-1.10
HPlasma membranes	1.16
HSmooth endoplasmic reticulum	1.16
HIntact oncogenic viruses	1.16-1.18
HMitochondria	1.19
HLysosomes	1.21
HPeroxisomes	1.23
HPlant viruses	1.30-1.45
HSoluble proteins	1.30
HRhino- and enteroviruses	1.30-1.45
HNucleic acids, ribosomes	1.60-1.75
HGlycogen	1.70
H	
H	
H	

H

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Table 3.2 Density and refractive indexes of sucrose

Density (g/cm3)	Refractive index	% by weight (w/v)	Molarity
H0.9982	1.3330	0	
H1.0021	1.3344	1	0.029
H1.0060	1.3359	2	0.059
1.0099	1.3374	3	0.089
1.0139	1.3388	4	0.119
1.0179	1.3403	5	0.149
1.0219	1.3418	6	0.179
1.0259	1.3433	7	0.210
1.0299	1.3488	8	0.211
1.0340	1.3464	9	0.272
1.0381	1.3479	10	0.303
1.0423	1.3494	11	0.335
1.0465	1.3510	12	0.367
1.0507	1.3526	13	0.399
1.0549	1.3541	14	0.431
1.0592	1.3557	15	0.464
1.0635	1.3573	16	0.497
1.0678	1.3590	17	0.530
1.0721	1.3606	18	0.564
1.0765	1.3622	19	0.597
1.0810	1.3639	20	0.632
1.0854	1.3655	21	0.666
1.0899	1.3672	22	0.701
1.0944	1.3689	23	0.735
1.0990	1.3706	24	0.771
1.1036	1.3723	25	0.806
1.1082	1.3740	26	0.842
1.1128	1.3758	27	0.878
1.1175	1.3775	28	0.914
1.1222	1.3793	29	0.951
1.1270	1.3811	30	0.988
1.1318	1.3829	31	1.025
1.1366	1.3847	32	1.063

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1.1415	1.3865	33	1.100
1.1463	1.3883	34	1.138
1.1513	1.3902	35	1.177
1.1562	1.3920	36	1.216
1.1612	1.3939	37	1.255
1.1663	1.3958	38	1.295
1.1713	1.3978	39	1.334
1.1764	1.3997	40	1.375
1.1816	1.4016	41	1.415
1.1868	1.4036	42	1.456
1.1920	1.4056	43	1.498
1.1972	1.4076	44	1.539
1.2025	1.4096	45	1.581
1.2296	1.4200	50	1.796
1.2575	1.4307	55	2.020
1.2865	1.4418	60	2.255
1.3163	1.4532	65	2.500

- Sucrose density fractions from Exercise 3.1

Procedure

1. Using the densities for sucrose given in Table 3.2 7, compute the density of each organelle found within the fractions collected by differential centrifugation.
2. Compare these values with those listed in Table 3.1. 8

Notes

It is possible to approximate the densities of the particles separated above, based on the linear gradient separation data. It would be better to perform an additional centrifugation using CsCl as the viscous media, but for our purposes, the density of the sucrose will be sufficient.

Exercise 3.3 - Equilibrium Density Gradient - Percoll

LEVEL II

Table 3.1 Densities of cell structures in sucrose

Table 3.3	Concentration	Density	Viscosity	Osmolality
Medium	(%w/v)	(g/ml)	(cP)	(mOs/kg H_2O)

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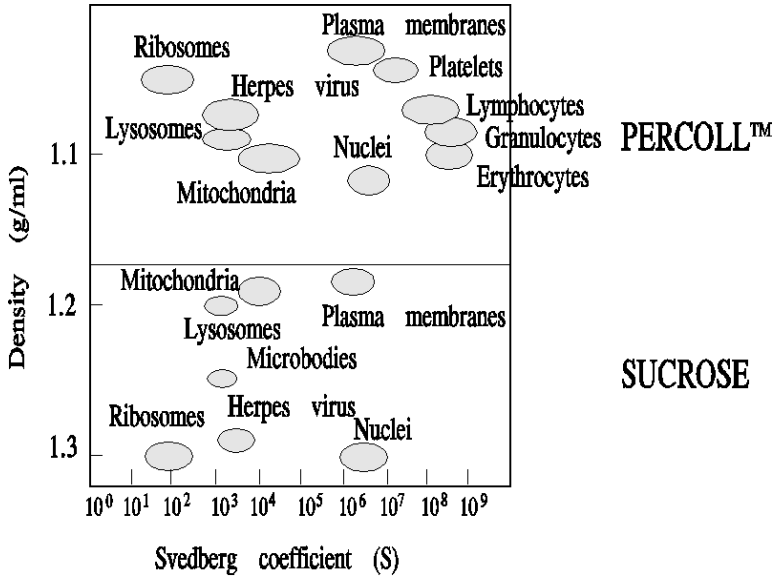
Sucrose	20	1.06	30	700
Metrizamide	30	1.16	2	260
Ficoll™	30	1.10	49	130
Percoll™	26	1.13	10	10

Figure 3.5 Svedberg Values in Percoll and Sucrose



Materials

- Ultracentrifuge and swinging bucket rotor
- Percoll
- NaCl solutions with the following osmolarities:
  - 200, 300 and 400 milliosmols
- Whole blood
- Hemacytometer
- Test tubes
- Colored density marking beads 9

Procedure 10

1. For your ultracentrifuge, obtain a series of centrifuge tubes containing appropriate volumes of Percoll starting at 1.08 g/ml density as follows:

   a. Percoll + colored marking beads.
   b. Percoll + 200 mOsm NaCl
   c. Percoll + 300 mOsm NaCl
   d. Percoll + 400 mOsm NaCl

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2. Use a hemacytometer to calculate the number of cells per ml of your blood sample. Carefully layer a suspension containing about 100 x 10^6 blood cells onto the gradients in tubes b-d.

   Centrifuge the four tubes for the equivalent clearance of Beckman 30.2 rotor at 35,000 xg for 15 minutes at 20° C.

3. Remove the tubes, fractionate into 0.5 ml fractions and count the number of cells in each fraction with a hemocytometer.
4. Graph the distance from the bottom of the tube vs. the number of cells in each fraction.
5. Overlay this graph with a comparison of distance vs. density of the medium, as determined by the position of the colored beads in your control tube.
6. Compare your results to Figure 3.5.

Notes

Pertoft and coworkers developed a synthetic, colloidal solution of polyvinylpyrrolidone coated silica, specifically designed for sedimentation centrifugation. This material is marketed under the trade name of Percoll. Table 3.3 give the characteristics of this medium, compared to several other density media, namely sucrose, metrizamide and Ficoll™.

Of particular interest is the fact that during centrifugation in a fixed angle rotor, Percoll™ will spontaneously form linear gradients, the shape of which is dependent upon rotor speed and time of centrifugation. Thus, it becomes a simple matter to establish a linear density gradient.

Figure 3.5 demonstrates a comparison of Percoll™ fractionation of cellular components to sucrose fractionation. This figure also presents, a standard way to comare components, by defining the relationship of density to the sedimentation coefficient for a cell or organelle. Note, that lymphocytes, granulocytes and erythrocytes have very similar sedimentation coefficients, but can be separated on the basis of density. Organelle separations are much easier to accomplish on Percoll™ density gradients than on sucrose gradients.

Exercise 3.4 - Low Speed Separaton of Cells

LEVEL II

Materials

- Sepracell-MN tubes 11
- Clinical centrifuge with fixed angle rotor
- Phase contrast microscope
- Whole Blood 12
- Phosphate Buffered Saline (PBS) + 0.1% (w/v) Bovine Serum
- Albumin (BSA)

Procedure

1. Collect at least 5.0 ml of blood into a Vacutainer containing EDTA as an anticoagulant.

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2. Add 5.0 ml of EDTA anti-coagulated whole blood to a Sepracell-MN tube. Mix gently by inversion.
3. Centrifuge at 2,000 xg for 10 minutes at room temperature with a fixed angle rotor. 13
   Decelerate the centrifuge slowly (do not use a brake).
4. Keeping the Sepracell-MN tube upright, insert a Seprapette into the tube and push until the MNC band (mononuclear cells) is displaced into the Seprapette. Do not collect more than 2.5 ml into the Seprapette. After the centrifugation of whole blood, there should be an opalescent compact band just below the meniscus of the tube. This bands contains the mononuclear cells (lymphocytes and monocytes) and platelets. Below this band is the plasma, while the erythrocytes and polymorphonuclear cells will form a dark band at the bottom of the tube.
5. Transfer the contents within the Seprapette by inverting it into a 15 ml conical centrifuge tube. Rinse the inside of the Seprapette with 2.5 ml of PBS and transfer the washing to the conical centrifuge tube.
6. Wash the mononuclear cells in the conical tube by adding 5 ml of PBS containing 0.1% BSA for a total volume of 10 ml and mix by inversion. Centrifuge at 300 xg for 10 minutes at room temperature.
7. Resuspend the mononuclear cells in 5.0 ml of PBS. Make a simple wet mount of the suspended cells and examine the purity of the separation using a phase contrast microscope.

Exercise 3.5 - Separation of Cells by Velocity Sedimentation

LEVEL III

It is possible to combine variations in density and size to allow for separation of cell populations within the earth's gravitational field, i.e. without a centrifuge. A technique devised by Miller and Phillips 14 uses a 3-30% gradient of fetal calf serum in phosphate buffered saline, coupled to the use of a specially designed sedimentation chamber.

The technique substitutes time for the expense of a centrifuge, but also keeps the cells from being subjected to increased gravitational forces. Several commercial applications of this technique are now available for the separation of blood cells within reasonable times (24 hours). Miller and Phillips used the technique to separate spleen cell suspensions and sheep erythrocytes. The technique is particularly valuable for the separation of cells of the hemopoietic system, prior to sub-culture or analysis.

Endnotes

---

1. Bean sprouts or alfalfa sprouts from a local supermarket may be substituted. They are inexpensive, non-poisonous and contain immature chloroplasts which separate well upon sucrose gradient centrifugation.
2. For optimal performance, the sucrose concentration should be determined by use of a refractometer. Pure sucrose, manufactured for use in molecular biology should be used, or it may be necessary to treat the sucrose for contaminating enzymes.
3. Modified from Terrance G. Cooper. *The Tools of Biochemistry.* John Wiley & Sons, New York, 1977. pp 347-352.
4. If only one laboratory section is used, two of each gradient should be prepared. The extra are used to

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balance the tubes in the centrifuge. It is not good practice to balance an ultracentrifuge with water or differing gradients since the density distribution will not be balanced, even if the total weight is.

5. This rotor has served the author well over many years, but is no longer available. The SW27 rotor has a k = 337 @ 25,000 RPM. Refer to Appendix F for conversion directions for other rotors.
6. Continuous flow spectrophotometry may be substituted, if available.
7. Data from *Techniques of Preparative, Zonal, and Continuous Flow Ultracentrifugation* by Owen Mitch Griffith, Ph.D. Applications Research Department, Spinco Division, Beckman Instruments, Inc. 1979, page 9.
8. Ibid, page 4.
9. These beads are a quick means of visually checking the density separation during formation of the Percoll gradient. They are available from Pharmacia, but are expensive. The use of the beads may be eliminated, with the corresponding elimination of Step 1a.
10. As reported in Pertoft, H., et al. "The Use of Density Gradients of Percoll® for the Separation of Biological Particles." in *Separation of Cells and Subcellular Particles* (H. Peeters, Ed.) Pergamon Press, Oxford, 1979.
11. Sepratech Corporation, Oklahoma City, OK 73127.
12. Sepracell-MN tubes were devised for human whole blood, but will work well on rabbit or other animal blood. The blood needs to be less than 12 hours old for best results, and no older than 36 hours.
13. If a fixed angle rotor is unavailable, use a swinging bucket, but increase the centrifugation time to 20 minutes.
14. Miller, R.G. and R.A. Phillips. "Separation of Cells by Velocity Sedimentation" J. Cell Physiol., 73:191-202. 1969.

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Chapter 4: Electrophoresis

Introduction

Figure 4.1 Hoefer SE 400 Sturdier™ Electrophoresis units

Electrophoresis may be the main technique for molecular separation in today's cell biology laboratory. Because it is such a powerful technique, and yet reasonably easy and inexpensive, it has become commonplace. In spite of the many physical arrangments for the apparatus, and regardless of the medium through which molecules are allowed to migrate, all electrophoretic separations depend upon the charge distribution of the molecules being separated. 1

Electrophoresis can be one dimensional (i.e. one plane of separation) or two dimensional. One dimensional electrophoresis is used for most routine protein and nucleic acid separations. Two dimensional separation of proteins is used for finger printing , and when properly constructed can be extremely accurate in resolving all of the proteins present within a cell (greater than 1,500).

The support medium for electrophoresis can be formed into a gel within a tube or it can be layered into flat sheets. The tubes are used for easy one dimensional separations (nearly anyone can make their own apparatus from inexpensive materials found in any lab), while the sheets have a larger surface area and are better for two- dimensional separations. Figure 4.1 shows a typical slab electrophoresis unit.

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When the detergent SDS (sodium dodecyl sulfate) 2 is used with proteins, all of the proteins become negatively charged by their attachment to the SDS anions. When separated on a polyacrylamide gel, the procedure is abbreviated as SDS--PAGE (for Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis). The technique has become a standard means for molecular weight determination.

Polyacrylamide gels are formed from the polymerization of two compounds, acrylamide and N,Nı -methylene- bis-acrylamide (Bis, for short). Bis is a cross-linking agent for the gels. The polymerization is initiated by the addition of ammonium persulfate along with either β-dimethyl amino-propionitrile (DMAP) or N,N,Nı,Nı,- tetramethylethylenediamine (TEMED). The gels are neutral, hydrophillic, three-dimensional networks of long hydrocarbons crosslinked by methylene groups.

The separation of molecules within a gel is determined by the relative size of the pores formed within the gel. The pore size of a gel is determined by two factors, the total amount of acrylamide present (designated as %T) and the amount of cross-linker (%C). As the total amount of acrylamide increases, the pore size decreases. With cross- linking, 5%C gives the smallest pore size. Any increase or decrease in %C increases the pore size. Gels are designated as percent solutions and will have two necessary parameters. The total acrylamide is given as a % (w/v) of the acrylamide plus the bis-acrylamide. Thus, a 7 1/2 %T would indicate that there is a total of 7.5 gms of acrylamide and bis per 100 ml of gel. A gel designated as 7.5%T:5%C would have a total of 7.5% (w/v) acrylamide + bis, and the bis would be 5% of the total (with pure acrylamide composing the remaining 2.5%).

Proteins with molecular weights ranging from 10,000 to 1,000,000 may be separated with 7 1/2% acrylamide gels, while proteins with higher molecular weights require lower acrylamide gel concentrations. Conversely, gels up to 30% have been used to separate small polypeptides. The higher the gel concentration, the smaller the pore size of the gel and the better it will be able to separate smaller molecules. The percent gel to use depends on the molecular weight of the protein to be separated. Use 5% gels for proteins ranging from 60,000 to 200,000 daltons, 10% gels for a range of 16,000 to 70,000 daltons and 15% gels for a range of 12,000 to 45,000 daltons. 3

Cationic vs anionic systems

In electrophoresis, proteins are separated on the basis of charge, and the charge of a protein can be either + or -- , depending upon the pH of the buffer. In normal operation, a column of gel is partitioned into three sections, known as the Separating or Running Gel, the Stacking Gel and the Sample Gel. The sample gel may be eliminated and the sample introduced via a dense non-convective medium such as sucrose. Electrodes are attached to the ends of the column and an electric current passed through the partitioned gels. If the electrodes are arranged in such a way that the upper bath is -- (cathode), while the lower bath is + (anode), and -- anions are allowed to flow toward the anode, the system is known as an anionic system. Flow in the opposite direction, with + cations flowing to the cathode is a cationic system.

Tube vs Slab Systems

Figure 4.2 Electrophoretic separations of proteins

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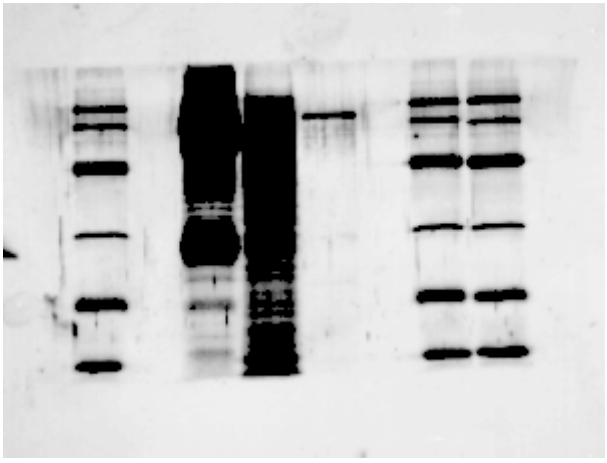

Two basic approaches have been used in the design of electrophoresis protocols. One, column electrophoresis, uses tubular gels formed in glass tubes, while the other, slab gel electrophoresis, uses flat gels formed between two plates of glass. Tube gels have an advantage in that the movement of molecules through the gels is less prone to lateral movement and thus there is a slightly improved resolution of the bands, particularly for proteins. It is also more economical, since it is relatively easy to construct homemade systems from materials on hand. However, slab gels have the advantage of allowing for two dimensional analysis, and of running multiple samples simultaneously in the same gel.

Slab gels are designed with multiple lanes set up such that samples run in parallel. The size and number of the lanes can be varied and, since the samples run in the same medium, there is less likelihood of sample variation due to minor changes in the gel structure. Slab gels are unquestionably the the technique of choice for any blot analyses and for autoradiographic analysis. Consequently, for laboratories performing routine nucleic acid analyses, and those employing antigenic controls, slab gels have become standard. The availability of reasonably priced commercial slab gel units has increased the use of slab gel systems, and the use of tube gels is becoming rare.

The theory and operation of slab gel electrophoresis is identical to tube gel electrophoresis. Which system is used depends more on the experience of the investigator than on any other factor, and the availability of equipment.

Figure 4.2 presents a typical protein separation pattern.

Continuous vs discontinuous gel systems

Figure 4.3 Schematic diagram of electrophoresis

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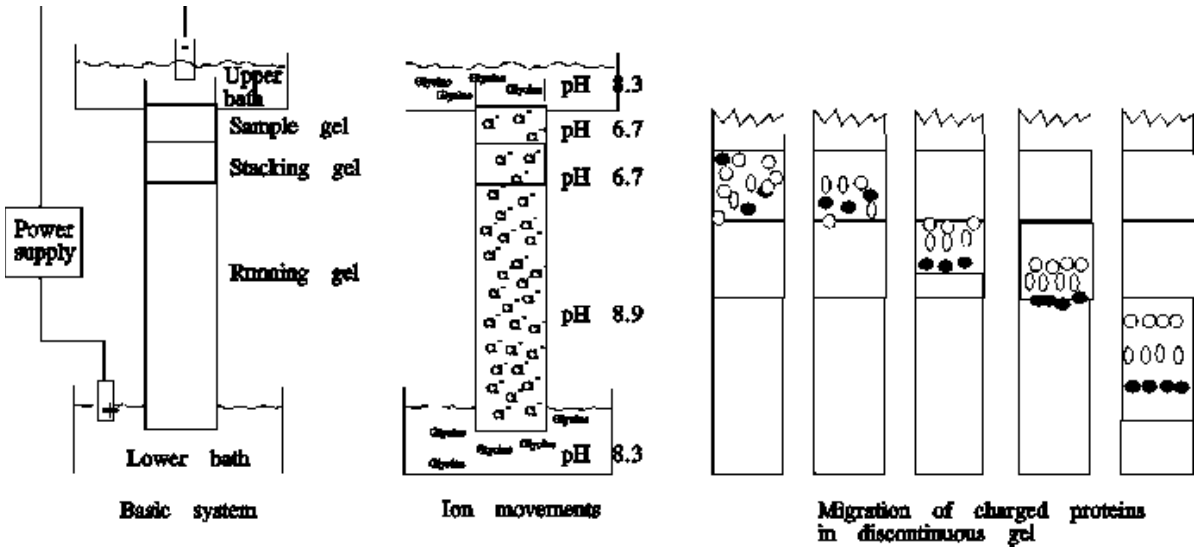

The original use of gels as separating media involved using a single gel with a uniform pH throughout. Molecules were separated on the basis of their mobility through a single gel matrix. This system has only occasional use in today's laboratory. It has been replaced with discontinous, [4] multiple gel systems. In multiple gel systems, a separating gel is augmented with a stacking gel and an optional sample gel. These gels can have different concentrations of the same support media, or may be completely different agents. The key difference is how the molecules separate when they enter the separating gel. The proteins in the sample gel will concentrate into a small zone in the stacking gel before entering the separating gel. The zone within the stacking gel can range in thickness from a few microns to a full millimeter. As the proteins are stacked in concentrated bands, they continue to migrate into the separating gel in concentrated narrow bands. The bands then are separated from each other on a discontinuous (i.e. disc) pH gel. [5]

Once the protein bands enter the separating gel, separation of the bands is enhanced by ions passing through the gel column in pairs. Each ioin in the pair has the same charge polarity as the protein (usually negative), but differ in charge magnitude. One ion will have a much greater charge magnitude than the proteins, while the other has a lesser charge magnitude than the proteins. The ion having a greater charge will move faster and is thus the leading ion, while the ion with the lesser charge will be the trailing ion. When an anionic system is employed, the Cl^- and glycinate (glycine as its acid derivative) ions are derived from the reservoir buffer (Tris-Glycine). The leading ion is usually Cl^- glycinate is the trailing ion. A schematic of this anionic system is shown in Figure 4.3. Chloride ions enter the separating gel first and rapidly move down the gel, followed by the proteins and then the glycinate ions. The glycinate ions overtake the proteins and ultimately establish a uniform linear voltage gradient within the gel. The proteins then sort themselves within this gradient according to their charge and size.

Agarose Gels

Figure 4.4 Agarose separation of cDNA

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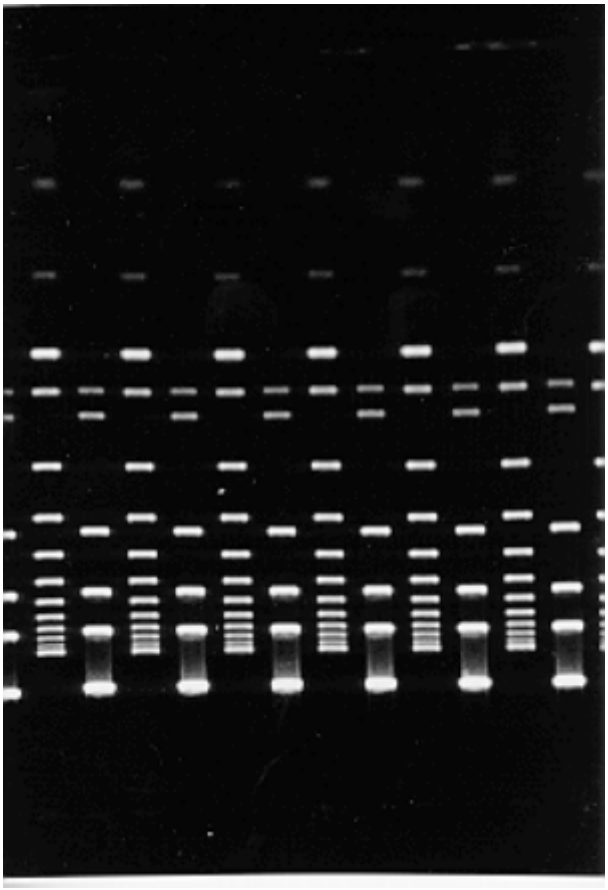

While acrylamide gels have become the standard for protein analysis, they are less suitable for extremely high molecular weight nucleic acids (above 200,000 daltons). In order to properly separate these large molecules, the acrylamide concentration needs to be reduced to a level where it remains liquid.

The gels can be formed, however, by the addition of agarose, a naturally linear polysaccharide, to the low concentration of acrylamide. With the addition of agarose, acrylamide concentrations of 0.5% can be used and molecular weights of up to 3.5 x 10^6 daltons can be separated. This is particularly useful for the separation of large sequences of DNA. Consequently, agarose-acrylamide gels are used extensively in today's genetic laboratories for the determination of gene maps. This chapter will concentrate on the separation of proteins, but Figure 4.4 demonstrates the separation of DNA fragments on an agarose gel.

Exercise 4.2 - Separation of Protein Standards: SDS-PAGE

LEVEL I

Figure 4.7 Rf vs log molecular weight (Laemmli)

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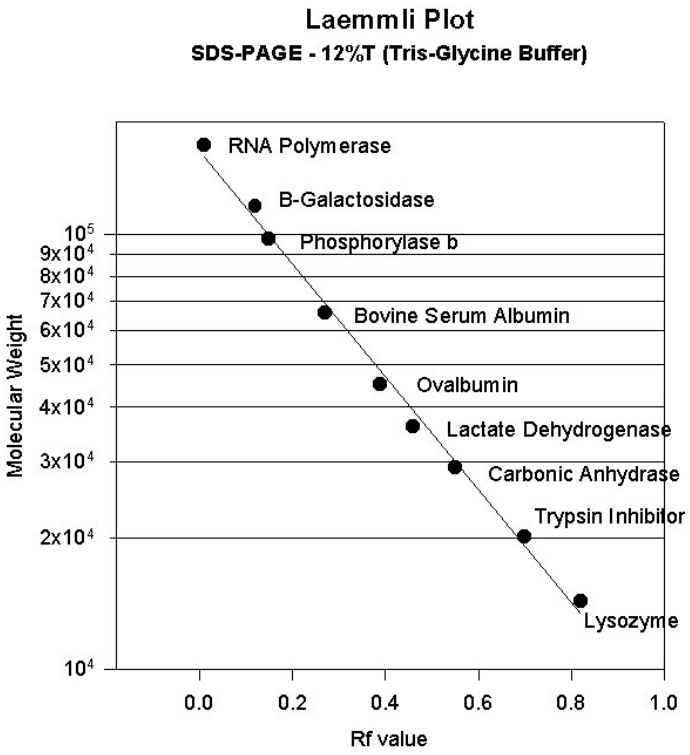

Materials

- 10% SDS-polyacrylamide gel from Exercise 4.1
- Protein standards 10
- 2X-SDS Sample Buffer
- 1X-SDS Electrophoresis Running Buffer (Tris-Glycine + SDS)
- 0.001% (w/v) Bromophenol Blue
- Micropipettes with flat tips for electrophoresis wells

Procedure

1. Remove the teflon combs from prepared gels by gently lifting the combs from the chamber. Rinse the wells (formed by the removal of the combs) with distilled water and drain off the water.
2. Fill the wells and the chamber with running buffer.
3. Prepare aliquots of a known protein standard by mixing equal parts of the protein standard with 2X sample buffer.
4. Using a micropipette, add the sample to the bottom of a well. 11
5. Add the same volume used in step 4 of SDS-sample buffer or bromophenol blue to a separate well. 12
6. Remove the gel from its casting stand and assemble it into the appropriate slab unit for running the electrophoresis. Be sure to follow the manufacturer's directions for assembly.
7. Pour a sufficient quantity of running buffer into both the lower and upper chambers of the electrophoresis apparatus until the bottom of the gel is immersed in buffer, and the top is covered, while the electrodes reach into the buffer of the upper chamber. Be careful not to disturb the samples in the wells when adding buffer to the upper chamber.
8. Assemble the top of the electrophoresis apparatus and connect the system to an appropriate power source.

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Be sure that the cathode (+) is connected to the upper buffer chamber.

9. Turn on the power supply and run the gel at 20 mA constant current per 1.5 mm gel.
   For example, if two gels are run, each having 1.5 mm spacers, the current should be adjusted to 40 mA. One gel with 1.5 mm spacers should be run at 20 mA, while a gel with 0.75 mm spacers should be run at 10 mA.
10. When the tracking dye reaches the separating gel layer, increase the current to 30 mA per 1.5 mm gel.
11. Continue applying the current until the tracking dye reaches the bottom of the separating gel layer (approximately 4 hours).
12. Turn off and disconnect the power supply. Disassemble the gel apparatus and remove the glass sandwich containing the gel. Place the sandwich flat on paper towels and carefully remove the clamps from the sandwich.
13. Working on one side of the sandwich, carefully slide one of the spacers out from between the two glass plates. Using the spacer or a plastic wedge as a lever, gently pry the glass plates apart without damaging the gel contained within.
14. Lift the bottom glass plate with the gel and transfer the gel to an appropriate container 13 filled with buffer, stain or preservative.
    The gel may at this point be used for Coomasie Blue staining (Exercise 4.3), silver staining (Exercise 4.4), enzyme detection (Exercise 4.7), Western blots (Exercise 4.9) or for more advanced procedur es, such as electroblotting or electroelution.
    If prestained protein standards were used, the gels may be scanned directly for analysis (Exercise 4.5 or Exercise 4.6). Place the gel into 50% methanol and gently rock the container for about 30 minutes prior to scanning. This can be accomplished by placing the gels into a flat dish and gently lifting the edge of the disk once every 30 seconds. There are commercially available rocker units for this purpose.
    If the gel is to be dried, use a commercial gel dryer, such as the Hoefer SE540 or SE 1160 Slab Gel Dryer. Follow the manufactuer's directions. Figure 4.6 demonstrates a dried and stained gel containing a series of proteins of known molecular weights.
15. Plot the **relative mobility** of each protein against the log of its molecular weight.

    Relative mobility is the term used for the ratio of the distance the protein has moved from its point of origin (the beginning of the separating gel) relative to the distance the tracking dye has moved (the gel front). The ratio is abbreviated as Rf. Molecular weight is expressed in daltons. Figure 4.7 presents a plot of the relative molecular weight of protein standards against the log of their molecular weight.

Exercise 4.3 - Coomasie Blue Staining of Protein Gels

LEVEL I

Materials

- Protein gel from Exercise 4.2
- 0.25% (w/v) Coomasie Brilliant Blue R 250 in methanol-water-glacial acetic acid (5-5-1), filtered immediately before use.
- 7% (v/v) acetic acid
- Commercial destaining unit (Optional)

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Procedure

1. Place a gel prepared as in Exercise 4.2 in at least 10 volumes of Coomasie Blue staining solution for 2-4 hours. Rock gently to distribute the dye evenly over the gel.
2. At the conclusion of the staining, wash the gels with several changes of water.
3. Place the gels into a solution of 7% acetic acid for at least 1 hour.
4. If the background is still deeply stained at the end of the hour, move the gels to fresh 7% acetic acid as often as necessary.
   If a commercial destainer is available, this will decrease the time required for stain removal. Follow the manufacturer's directions for use of the destainer. 14
5. Place the gels into containers filled with 7% acetic acid as a final fixative.
6. Photograph the gels or analyze the gels spectrophotometrically.

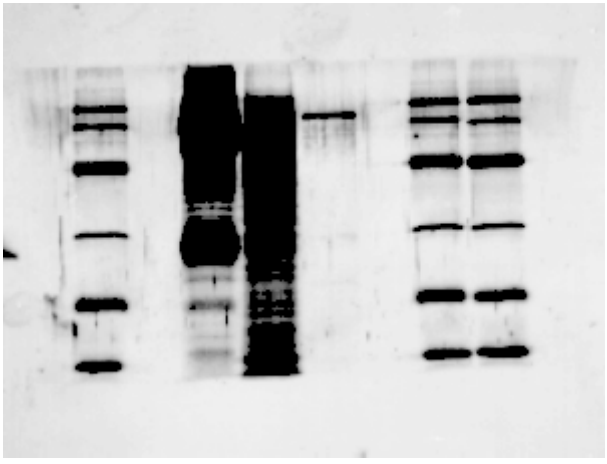
Notes

Coomasie Brilliant Blue R 250 is the most commonly used staining procedure for the detection of proteins. It is the method of choice if SDS is used in the electrophoresis of proteins, and is sensitive for a range of 0.5 to 20 micrograms of protein. Within this range, it also follows the Beer-Lambert law and thus can be quantitative as well as qualitative. The major drawback is the length of time for the procedure and a requirement for destaining. Overstaining results in a significant retention of stain within the gel, and thus a high background stain, which might obliterate the bands. The length of time for staining must be carefully monitored, and can range from 20 minutes to several hours. If maximum sensitivity is desired, one should try 2 hours for a 5% gel and 4 hours for a 10% gel. Destaining must be monitored visually and adjusted accordingly.

Exercise 4.4 - Silver Staining of Gels

LEVEL I

Figure 4.8 Silver stained protein gel



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Materials

- Protein gel from Exercise 4.2
- 45% (v/v) Methanol + 12% (w/v) acetic acid
- 5% (v/v) Methanol + 7% (w/v) acetic acid
- 10% Glutaraldehyde
- 0.01M Dithiothreitol
- Silver nitrate solution
- Sodium citrate / formaldehyde
- Kodak Farmer's Reducer or Kodak Rapid Fixer

Procedure

1. Fix gels by gently rocking them in a solution of 45% methanol / 12% acetic acid until the gels are completely submerged. Fix for 30 minutes at room temperature.
2. Remove the fixative and wash 2x for 15' each with 5% ethanol / 7% acetic acid. (Gels thicker than 1 mm require longer washing.)
3. Soak the gels for 30 minutes in 10% glutaraldehyde.
4. Wash 3x with deionized water, 10 minutes each.
5. Place in dithiothreitol for 30 minutes.
6. Place in silver nitrate solution for 30 minutes.
7. Wash for 1 minute with deionized water.

   Dispose of used silver nitrate solution immediately with continuous flushing. This solution is potentially explosive when crystals form upon drying.

8. Place in sodium citrate / formaldehyde solution for 1 minute.
9. Replace the sodium carbonate/formaldehyde solution with a fresh batch, place gels on a light box and observe the development of the bands. Continue to rock gently as the gel develops.
10. When the desired degree of banding is observed (and before the entire gel turns black), withdraw the citrate / formaldehyde solution and immediately add 1% glacial acetic acid for 5 minutes.
11. Replace the glacial acetic acid with Farmer's reducer or Kodak Rapid Fixer for 1 minute. Remove Farmer's reducer and wash with several changes of deionized water.
12. Photograph or scan the gel with a densitometer. Figure 4.8 demonstrates a typical silver stained protein gel.
13. For storage soak the gel in 3% glycerol for 5 minutes and dry between dialysis membranes under reduced pressure at 80-82° C for 3 hours. Alternatively, place the wet gel into a plastic container (a storage bag will do) and store at room temperature. If desired, the gels may be dried between Whatman 3MM filter paper for autoradiography, or dried using a commercial gel dryer.

Exercise 4.5 - Documentation

LEVEL I

Figure 4.8 Silver stained protein gel

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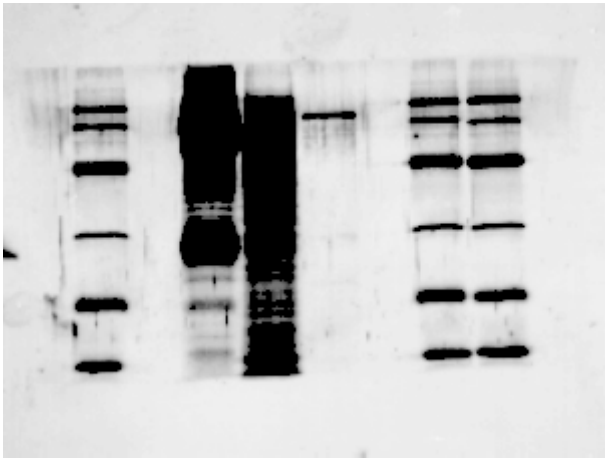

Materials

- Polaroid camera (Fotodyne Foto/Phoresis I or equivalent) 15
  or
- 35 mm camera equipped with macro lens
- Stained gel

Procedure

1. Photograph the gels.
2. Use the photographs or negatives to measure the distance from the point of protein application (or for two gel systems, the line separating the stacking and separating gels) to the final location of the tracking dye near the bottom of the gel. 16
3. Measure the distance from the point of origin to the center of each band appearing on the gel.
4. Divide each of the values obtained in Step 3 by that obtained in Step 2 to obtain the relative mobility (the Rf value) for each band.
5. Using either the graph of Rf values and molecular weights from Exercise 4.2, or Figure 4.8, compute the molecular weights of each band.

Optional

Scan the negative with a densitometer and compute Rf values based on the distances from the point of origin to the peak tracing for each protein band. Integration of the area of each peak will yield quantitative data as well as the molecular weight.

Exercise 4.6 - Direct Densitometry of Gels

LEVEL II

Figure 4.8a Model 1312 Densistometer

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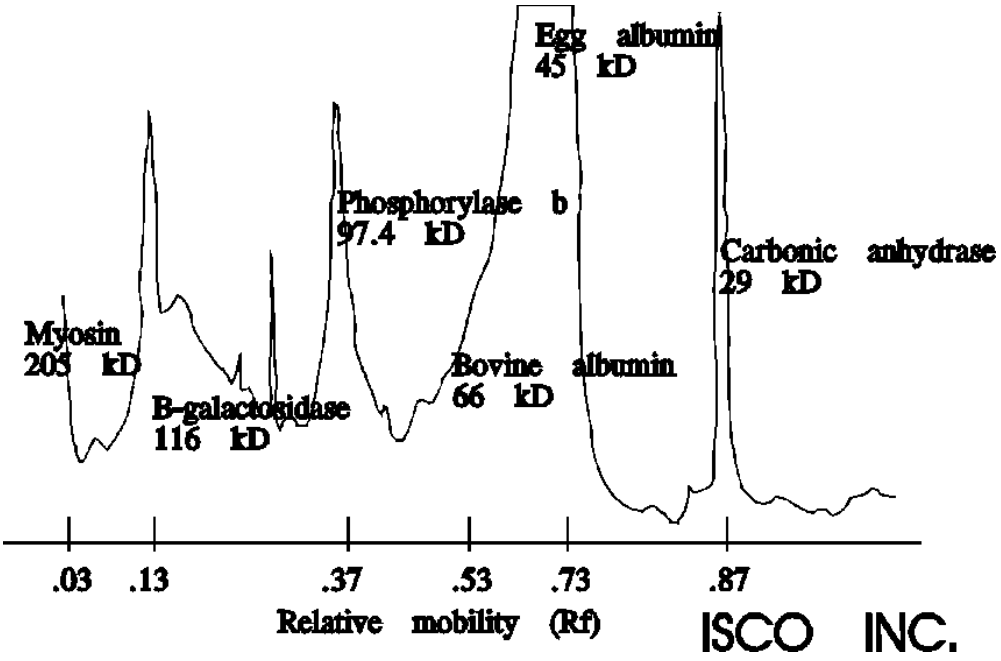
Figure 4.9 Protein standard scan from Model 1312



Materials

- Densitometer 17
- Washed and stained gel from Exercise 4.2

Procedure

1. Using the scanning cuvette supplied with your densitometer/scanner, calibrate the scanner and choose the proper scanning mode and wavelength filter for your application. For example, for the Isco Model 1312,

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use the instrument on Automatic Scan Mode with a 620 nm filter for Coomasie blue, or a 580 nm filter for silver stained gels.

2. Place the cuvette holder on the lab bench and pour about 10 ml of buffer or acetic acid into the cuvette. Unfixed gels still in the buffer that you used to wash them will work best for this purpose. If the gels have been fixed, be careful of spills and minimize contact with the cuvette to prevent corrosion of the cuvette and long term etching of the glass plates.
3. Place the stained gel into the buffer in the cuvette and orient the gel so that the lanes are vertical as you look at them, with the origin at the top and the front at the bottom. Be careful to gently remove any trapped air bubbles beneath the gel by gently lifting the gel with a blunt glass rod.
4. Very carefully lay the top glass plate onto the gel to hold it flat. The plate should be placed on the gel at an angle, with the two black knobs on the right, and slowly lowered onto the gel. Again, be careful to prevent trapping air bubbles beneath the glass plate.
5. Adjust the scan rate to match that of the recorder (try an initial scan rate of 300 cm/hr). Place the cuvette onto the optical reading device, and manually adjust the position of the gel so that the optical reading head of the scanner is placed just before the origin point of the gel lane to be scanned, in an area where there are no bands. Adjust the baseline of the scanner and recorder to zero.
6. Press and hold the START/STOP button until the scan begins. Continue the scan until the optical head returns to its original position. Be careful that the head does not strike one of the knobs on the top glass plate during operation. This will result in possible damage to the optical unit.
7. When all lanes are scanned, return the gel to storage and carefully clean the gel cuvette.
8. Calculate the Rf values for each peak and determine the molecular weights of the proteins located within each peak. Figure 4.9 presents a typical scan of protein standards.

Optional

Modern scanners are equipped with capability to output analog data directly to various computer data management systems, while converting the data to digital information. In addition to storing the scan as a digital reading, these programs can be used to integrate the area under the curves for each peak and thereby yield quantitative data. If such a capability is present on your system, calculate the amount of each protein found within the major peaks. 18

Exercise 4.7 - Separation of LDH Isozymes from Serum

LEVEL II

Figure 4.10 Preparation rack for tube gels

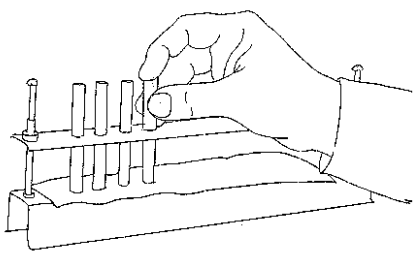

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Figure 4.11 Filling gel tubes

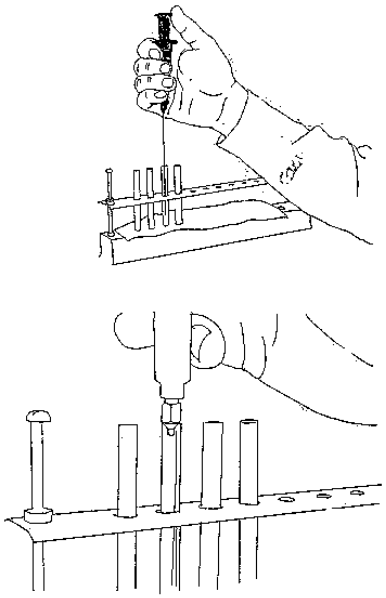

Figure 4.12 Assembling tubes and bath chambers

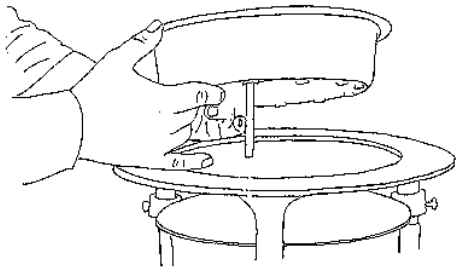

Figure 4.13 LDH isozyme separation

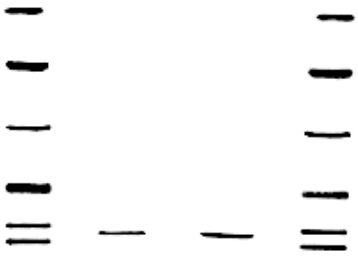

Image courtesy of Beckman Instruments

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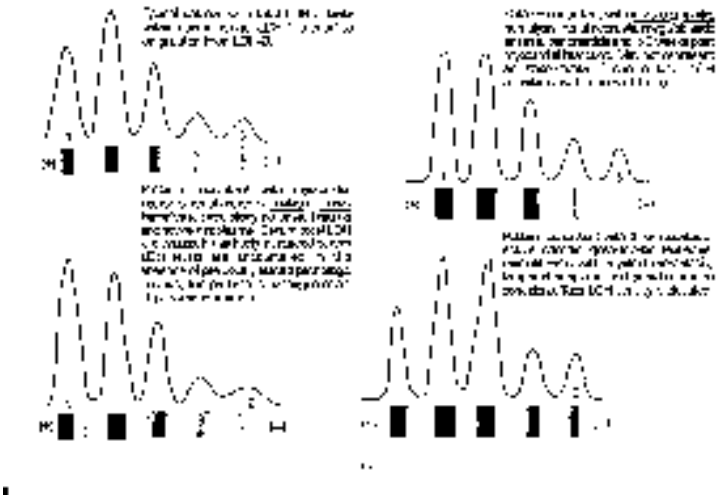
Figure 4.14 Densitometry tracings of LDH patterns



Materials

- 1.0 M Tris-HCl pH 8.8
- Stock 30%T:0.8%C Acrylamide
- 10% (w/v) Ammonium persulfate
- 40% (w/v) Sucrose
- Tris-Glycine Buffer
- LDH Stain: Must be prepared just prior to use. For use, combine 8 parts A to 1 part B. Solution A:

60% Sodium Lactate	2.4 ml
Nitro blue tetrazolium	0.03 gm
Phenazine Methosulfate	0.003 gm
Sodium phosphate buffer to make	100 ml

- Solution B:

0.5% (w/v) NAD (use 5 mg/ml H_2O)

- Non-heparanized hematocrit tubes and centrifuge
- Blood lancets and alcohol for swabbing 19

Procedure

The following directions are given for the preparation of tube gels. Many laboratories continue to use tube gels. The directions can be readily modified to use slab gels as detailed in Exercise 4.1 and Exercise 4.2

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1. Assemble the appropriate sized glass tubes and a preparation rack. These may be cut from standard glass tubing or available commercially.
2. Insert the glass tubes in the preparation rack by laying a strip of Parafilm approximately 14" long on top of the black grommets located on the base of the preparation rack (Figure 4.10). Place Parafilm so that it just covers the first grommet and allow an approximate 2" overhang at the opposite end. Beginning at the first grommet, insert the tube through the hole in the upper section of the rack and press the tube down into the grommet, pushing the Parafilm ahead to form a tight leak-proof seal. Place your finger under the grommet and seat the tube and Parafilm against your finger. Do not stretch or pull the Parafilm while inserting the tubes. Leave a small amount of slack as you proceed from one tube to the next.
3. Prepare the separating gel (5% acrylamide) by mixing the following:

Stock Acrylamide	3.0 ml
Tris-HCl buffer	3.0 ml
Ammonium persulfate	6.0 ml
TEMED	20 µl

4. Mix slighty more than 1 ml of solution for each tube being prepared. Once the reagents are mixed, complete the next two steps quickly. The mixture will begin to polymerize in approximately 10 minutes. Rinse syringe, beakers, and other implements after use to prevent the gel solution from solidifying on them.
5. Using a 10 cc syringe with a Loading Syringe Stub Needle, fill the gel tubes to within approximately 2 cm of the top with the separating gel mixture (Figure 4.11). Expel any remaining solution from the syringe when done.
6. Insert the syringe with stub needle into each gel tube as far as it will go; draw off as much gel solution as possible. This procedure will result in gels of uniform height. After removing excess gel solution from the gel tubes, rinse the syringe and stub needle. Carefully add a small layer of Tris-HCl Buffer to the top of the gel, without disturbing the gel itself.
7. Allow the separating gel solution to stand undisturbed for 30 minutes during polymerization.

The following step involves the use of human blood. Extreme caution must be taken to guard against the dangers of infection. Use disposable lancets, wear gloves at all times and dispose of sharps as indicated by the instructor. Alternatively use blood from a laboratory animal.

8. Using a non-heparanized hematocrit capillary tube, obtain a sample of blood. Centrifuge the blood sample in a hematocrit head of a clinical centrifuge to separate the formed elements from the serum/plasma. A standard hematocrit tube contains just the right serum for a single gel tube analysis. Alternatively, blood can be centrifuged in regular centrifuge tubes, if there is a sufficient quantity of blood.
9. For each blood sample, prepare a 1:10 dilution of the sample by combining 1 part of sample serum with 9 parts of sucrose. Prepare at least 100 µ l of diluted serum for each specimen, enough for two gels.
10. Remove the storage solution from three gel tubes by inverting the gel tubes over a layer of absorbent paper. Shake the tubes abruptly once or twice in a downward motion to remove all the buffer. While the gel tubes are still inverted, blot the ends of the tubes to remove any remaining liquid.
11. Apply 50 µ l of diluted serum to each of two gel tubes. Do not touch the surface of the gel, but allow the

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sample to flow onto its surface.

12. Layer each gel tube with tris-glycine buffer to completely fill the tube. Be extremely careful not to disturb the serum layer.
13. Remove gel tube #1 from the preparation rack. Moisten the upper end of the tube with a little water and insert sample end up into the bottom of the bath tube adapter (position 1 --). Push the tube in so that its upper edge is flush with the upper edge of the adapter. Place all gel tubes into the bath in this manner. Observe the numbers on the upper bath lid and use them to identify the corresponding samples from the preparation rack. Handle the gel tubes with care to prevent mixing of sample and buffer layer. Use the hollow plastic bath stoppers to plug any empty tube adapters.
14. Pour enough tris-glycine buffer into the lower bath so that the bath is approximately half full.
15. Assemble the upper and lower chambers, ensuring that the lower portions of the gel tubes are immersed in the lower bath and that all trapped air bubbles are removed from the ends of the tubes.
16. Carefully pour additional tris-glycine buffer into the upper bath, so that the level is sufficient enough to make contact with the cathode when it is inserted (Figure 4.12). Be particularly careful not to disturb the buffer inside the gel tubes. Again, check the gel tubes to insure that there are no air bubbles trapped in the upper part of the gel tubes.
17. Place the cover onto the upper bath and connect the electrodes to the baths and the power supply.
18. Turn on the power source and, if necessary, allow it to warm up. Check the bath to insure that the polarity is normal. Connect the safety interlock jack to the pins on the bath. Adjust the power source to deliver 5 milliamps of current per gel tube in the bath. For example, if 6 gel tubes are being run, the total current should be 30 milliamps.
19. Continue electrophoresis for 20 minutes or until a clearly defined albumin band is seen near the bottom of the tube. If prestained protein markers are used in the standard, the timing can be precisely controlled by visually checking its progress.
20. When electrophoresis is complete, disconnect the safety inter lock and turn the power source off. Pour the upper bath buffer into a storage container. Place the upper bath on the U-stand and remove the first gel tube.
21. Remove the separating gel for staining. Fit the 10 cc syringe with the blunt-tipped gel removing needle and half fill the syringe with water. While holding the gel tube with one hand, carefully insert the needle at the sample application end of the tube, between the inside wall of the tube and the gel. While slowly pushing the needle in and keeping it flat against the tube wall to avoid scratching the gel, force a stream of water through the needle and rotate it completely around the circumference of the gel. Remove the needle and insert it from the other end of the tube (the separating gel end) using the same technique described above. Once the needle is inserted and rotated completely, the gel will come loose and slide from the tube.
22. Rinse the gel with distilled or deionized water to remove any enzymes on the surface and place the gel in a stoppered test tube.
23. Repeat steps 21-22 for the rest of the tubes. Label each gel carefully.
24. Place 2 ml of freshly prepared LDH stain solution into 10 x 75 mm amber tubes and add a gel to each tube. Keep stoppered and away from light.
25. Incubate the gels in stain solution at 37 ° C for 60 minutes.
26. When the color of the bands has developed, drain off the stain solution from the tubes and fill them with 7% acetic acid. Continue to protect the gels from light for one hour. At the end of this time, the acetic acid will have inhibited further color development and preserved the protein gels.
27. Transfer each gel to a clear glass test tube containing fresh 7% acetic acid and stopper the tube. The gels are now ready for photography or densitomer quantitation. Figure 4.13 and 4.14 demonstrate LDH

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separation patterns for a normal individual and a comparison with a pattern consistent with myocardial necrosis.

28. If the gels are scanned, integrate the area of each band and calculate the amount of each isozyme as a % of the total LDH present.

Notes

The term isozyme was introduced by C.L. Markert and F. Moller in 1959 20 to describe multiple enzyme forms with similar or identical substrate specificity, and occurring within the same organism. Markert later proposed to modify the term by such adjectives as allelic, nonallelic, multimeric, conformational, and conjugated. These adjectives imply a broader definition of the term isozyme and thus include many genetic variations, as well as physiological modifications of the protein structures.

Serum lactic dehydrogenase (LDH) is an ideal enzyme for the analysis of isozymes, and is also a model system for electrophoretic analysis. The enzyme actually consists of five electrophoretically separable isoenzymes, identified as LDH--1, LDH--2, LDH--3, LDH--4, and LDH--5 in the order of their relative rates of electrophoretic migration; LDH--1 migrates most rapidly. Since these isoenzymes are usually associated with characteristic tissue or organ sources, their relative concentrations in serum may provide useful information in the differentiation of diseases of various body systems, such as myocardial infarction, liver necrosis, pulmonary infarct, primary muscle dystrophy, pernicious anemia, and malignancy. Consequently several easily available commercial kits can be used for their analysis.

LDH isozymes are important indicators of cellular differentation as well. Analysis of the peptide synthesis for LDH isozymes presents what is now a classical analysis of differential gene activity.

LDH1 is composed of a single peptide species A, while LDH5 is composed of the single peptide species B. LDH2-4 are the permutations of combining species A and B into a functional tetramer. Thus, whether LDH1 or LDH5 are synthesized depends entirely on the gene transcription and translation for species A and B respectively. If both are turned on and are equimolar within the cell, then LDH3 will be the predominant form (AABB). In short, the LDH tetramer is a self-assembling molecule that depends upon the concentration of reactants for its tetrameric form. The distribution of isozymes can thus be analyzed statistically to determine the extent to which gene A and/or gene B are functioning.

Exercise 4.8 - Western Blots

LEVEL III

Materials

- Blot Cell
- BA 83 0.2 µ m pore nitrocellulose sheets
- Buffer, PBS-Tween 20
- Antigenic proteins, antibodies, and horseradish peroxidase labeled antiglobulins

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Procedure 21

1. Run an electrophoretic separation of known antigenic proteins according to the procedures in Exercise 4.1 and 4.2.
2. Draw a line 0.5 cm from the top edge of an 8 x 10 cm nitrocellulose sheet and soak it in blot buffer for about 5 minutes.

   Nitrocellulose is both fragile and flammable and easily contaminated during handling. Wear gloves which are prewashed.

   When soaking the nicrocellulose wet first one side and then turn the sheet over and wet the other, to prevent trapping air within the filter.

3. Place 200 ml of blot buffer into a tray and add a piece of filter paper slightly larger than the electrophoretic gel from Step 1.
4. Remove the gel from the electrophoresis chamber after the proteins have been separated, and place the gel into the tray containing the filter paper. Do not allow the gel to fall onto the paper, but place it next to the paper in the tray.
5. Gently slide the gel onto the top of the filter paper. Keep the stacking gel off of the paper until the last moment, since it tends to stick and make repositioning difficult.
6. Holding the gel and the filter paper together, carefully remove them from the tray of blot buffer and transfer the paper and gel to a pad of the blot cell with the gel facing up.
7. Transfer the nitrocellulose sheet (ink side down) onto the top of the gel and line up the line drawn on the sheet with the top of the stacking gel.

   Once the gel and nitrocellulose touch they can not be separates.

8. Roll a glass rod across the surface of the nitrocellulose to remove any air bubbles and insure good contact between the gel and nitrocellulose.
9. Lay another sheet of wet filter paper on top of the nitrocellulose creating a sandwich of paper-gel-nitrocellulose-paper, all lying on the pad of the blot cell.
10. Add a second pad to the top of the sandwich and place the entire group inside of the support frame of the blot cell, and assemble the blot cell so that the nitrocellulose side of the sandwich is toward the positive terminal.
11. Check that the buffer levels are adequate and that the cooling water bath is adjusted to at least 5° C. Subject the gel to electrophoresis for 30 minutes with the electrodes in the high field-intensity position. Follow the manufacturer directions during this phase. Failure to closely monitor the electrophoresis buffer or temperature can result in a fire. Use a circulating cold bath appropriate to the apparatus and hold the voltage to a constant 100 vdc.
12. Upon completion of the electrophoresis (timed according to manufacturer's directions), turn off the power and disassemble the apparatus. Remove the blot pads from the sandwich and remove the filter paper from the nitrocellulose side.
13. Place the sandwich, nitrocellulose side down, onto a glass plate and remove the other filter paper.
14. Use a ball point pen to outline the edges of the separating gel onto the nitrocellulose, including the location of the wells. Carefully lift the gel away from the nitrocellulose and mark the locations of the pre-stained molecular weight standards as the gel is peeled away. Peel the gel from the separating gel side, not the stacking gel.

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15. Wash the blot (the nitrocellulose sheet) at least four times with 100 ml of PBS-Tween 20 for five minutes each on a rocking platform.
16. Cut the blot into 0.5 cm strips.
17. Inactivate sera containing positive and negative antibody controls to the antigens under examination by treating at 56 ° C for 30 minutes. Make dilutions of 1:100 and 1:1000 of the controls with PBS-Tween 20.
18. Place 3 ml of the diluted sera or controls onto a strip from Step 16 and incubate for 1 hour at room temperature while continuously rocking the sample.
19. Wash the strips four times for 5 minutes each with 10 ml quantities of PBS-Tween 20. The first wash should be done at 50° C but the last three may be done at room temperature.
20. Add 3 ml of horseradish peroxidase-labeled antiglobulin, optimally diluted in PBS-Tween and incubate at room temperature for 1 hour with continuous agitation.
21. Wash the strips four times for 5 minutes each with PBS- Tween 20 and one more time with PBS only.
22. Remove the PBS and add 5 ml of substrate solution. Positive reaction bands usually appear within 10 minutes. Stop the reaction by washing with water. Refer to Figure 4.15 for a comparison.

Notes

One of the more difficult tasks of electrophoretic separations is the identification of specific bands or spots within a developed gel. As observed with LDH isozymes, one method of doing this is to react the bands with an enzyme substrate that can be detected colorimetrically.

As a rule, however, most peptides are denatured during electrophoresis, and, of course, nucleic acids have no enzyme activity. The methods employed for identifying non- enzymatic proteins and nucleic acids have been termed Western for immunoblotting of proteins, Southern for techniques using DNA probes Northern when using RNA probes. The probes are radioactive complimentary strands of nucleic acid. The first of these techniques was the Southern, named for the developer of the procedure, Edward Southern. Northern and then Western blots were named by analogy.

Blotting techniques first develop a primary gel: protein on acrylamide; or DNA/RNA on agarose. The gel patterns are then transferred to nitrocellulose membrane filters and immobilized within the nitrocellulose membrane. This process of transfer to an immobilizing substrate is where the term blotting originated. The process is widely used in today's laboratories because the immobilization allows for extensive biochemical and immunological binding assays that range from simple chemical composition to affinity purification of monospecific antibodies and cell- protein ligand interactions.

In practice, the electrophoresis gel is sandwhiched between two layers of filters, two foam pads (for support) and two layers of a stainless steel mesh. This entire apparatus can be submerged in a buffer and transfer allowed to occur by diffusion (yielding two blots, one on each filter), or can be arranged in an electro-convective system so that transfer occurs in a second electrophoretic field.

Once the transfer has occurred, the blots can be probed with any number of specific or non-specific entities. DNA can be probed, for example, with cDNA or even a specific messenger RNA to identify the presence of the gene for that message.

Endnotes

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1. We refer to molecular orientation. It is also possible to separate whole cells or organelles on the basis of their surface charge.
2. Also known as sodium lauryl sulfate or SLS.
3. Guidelines given by John A. Smith, in *Current Protocols in Molecular Biology*, Section II, 10.2.2 "Electrophoretic Separation of Proteins". Greene Publishing Associates, Brooklyn 1987.
4. The term disc is applied to discontinuous gels, but unfortunately has two meanings when applied to electrophoresis. Some investigators continue to use an older reference to the discoidal front found in tube gels, and thus use disc interchangeably with the terms column or tube. Modern usage of the term refers to the term being applied to a discontinuous pH system which may be run in a tube or slab configuration.
5. If a continuous pH gradient is employed, the stacking gel is eliminated, the buffers are different, and the technique is referred to as zone electrophoresis. If the molecules being separated are allowed to migrate to a particular zone due to a pH gradient in the gel, the procedure becomes known as electrofocusing.
6. These can be easily manufactured from 22 gauge needles with the points ground off. Never use pointed needles when working with acrylamide!
7. Alternatively, add a stirring bar to the flask and stir on a magnetic mixer at low speed. It is important that the solution be stirred slowly so as to not introduce air bubbles.
8. If problems are encountered using water at this step, water saturated isobutanol may be substituted. To aid even further, a little Oil Red O dye can be added to the isobutanol for visual distinction. Since the alcohol is less dense than water, it layers more readily without mixing with the gel. It must be thoroughly rinsed out before adding the stacking gel, however.
9. Teflon combs come in a variety of configurations for sample volume and number of lanes (3,5,10,15 or 20). Use a comb suitable for the number of samples to be run.
10. Prestained molecular weight standards are available from several sources; BRL, Rockville, Md, Bio-Rad or Sigma Chemical Co, St. Louis.
11. The SDS/sample buffer contains glycerol and consequently the sample will have a higher density than the SDS/separating buffer. It is best to use a flat tipped micropipette designed specifically for this application, but any flat tipped syringe needle will also work. The volume of sample will depend on the size of the wells, but typically is 25-100 µl. Some experimentation will be necessary to obtain the proper volume.
12. In some applications, the bromphenol blue is added to the SDS/sample buffer and thus this step is unnecessary. Bromphenol blue is known as "tracking" dye and is used to monitor the progress of the electrophoresis separation. A drop of 0.1% phenol red may be substituted. Alternatively, prestained protein standards may be used without a tracking dye, although this is not recommended.
13. 10x10x2 glass baking dishes are useful for this purpose, or ordinary refrigerator containers of appropriate size. While the former will need a covering of plastic wrap, the latter come with lids.
14. If tube gels are used, the apparatus used for separation can be modified for stain removal if slightly larger tubes can be fitted. Place a gel that has been washed in water and 7% acetic acid (1 hr.) into a tube that has had a 0.5 cm gel plug formed in the tip. Place the gel into the tube with the origin up, that is away from the plug, and fill the tube with 7% acetic acid. Insert the tube into the appropriate apparatus and fill both upper and lower chambers with 7% acetic acid. With the upper pole as the cathode, apply a 15 milliamp current per tube. The stain will wash into the lower bath and the progress can be monitored visually.
15. Fotodyne Inc. New Berlin, Wisconsin.
16. Measurements can be made easier if the image of the gel is first enlarged such that the distance from the point of origin to the front is 10 cm on the final print. This can be done with a photographic enlarger, and

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a print may not be required.

17. Directions given for Isco Model 1312 Gel Scanner (Isco, Inc. 4700 Superior, Lincoln, NE 68504).
18. Alternatively, a cruder yet effective method, is to cut the peaks from the paper tracing and weigh the paper for each peak. This must be done at one period of time to prevent changes in the weight due to humidity. The technique is surprisingly accurate, however. This process can also be used with photographs of the gels, without the use of an expensive gel scanner.
19. Human serum works well for this exercise, and students may use their own blood. To avoid the obvious problems in obtaining safe samples of human blood products, substitute fresh sera from laboratory animals. A rabbit or rat will work, but the LDH patterns will be different than those given in this exercise.
20. C.L. Markert and F. Moller 1959.
21. From Enzyme-Linked Immunoelectrotransfer Blot Technique (Western Blot) for Human T-Lymphotropic Virus Type III/Lymphadenopathy-Associated Virus (HTLV-III/LAV) Antibodies VC.W.Tsang, K.Hanclck, M. Wilson, D.F. Palmer, S.D. Whaley, J.S. McDougal, S. Kennedy. Immunology Series No. 15 Procedural Guide. U.S. Dept. of Health and Human Services. December 1986.

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Chapter 5: Enzymes

Introduction

Cells function largely because of the action of enzymes. Life is a dynamic process that involves constant changes in chemical composition. These changes are regulated by catalytic reactions, which are regulated by enzymes. At one time, the cell was actually concieved of as a sac of enzymes. It was believed that if we knew all of the reactions and their rates of action, we could define the cell, and indeed, life itself. Few biologists continue to think of this as a simple task, but we know that life as we know it could not exist without the function of enzymes. Ideally, we would examine enzymes within an intact cell, but this is difficult to control. Consequently, enzymes are studied **in vitro** after extraction from cells. In this exercise, we will extract the enzyme tyrosinase and study its kinetic parameters. It is only one of thousands of enzymes working in concert within cells, but it is one but which readily demonstrates the main features of enzyme kinetics.

Since all enzymes are proteins, and proteins are differentially soluble in salt solutions, enzyme extraction procedures often begin with salt (typically, ammonium sulfate) precipitation. On the simplest level, proteins can be divided into albumins and globulins on the basis of their **solubility** in dilute salts. Albumins are considered to be soluble while globulins are insoluble. Solubility is relative, however, and as the salt concentration is increased, most proteins will **precipitate**.

Thus, if we homogenize a tissue in a solution that retains the enzyme in its soluble state, the enzyme can be subsequently separated from all insoluble proteins by centrifugation or filtration. The enzyme will be impure, since it will be in solution with many other proteins. If aliquots of a concentrated ammonium sulfate solution are then added serially, individual proteins will begin to precipitate according to their solubility. By careful manipulation of the salt concentrations, we can produce fractions which contain purer solutions of enzymes, or at least are enriched for a given enzyme. Fortunately, absolute purity of an enzyme extract is seldom required, but when it is, the fractions must be subjected to further procedures designed for purification (such as electrophoresis and/or column chromatography).

In order to determine the effectiveness of the purification, each step in the extraction procedure must be monitored for enzyme activity. That monitoring can be accomplished in many ways, but usually involves a measurement of the decrease in substrate, or the increase in product specific to the enzyme.

It is important to remember that enzymes act as catalysts to a reaction and that they affect only the reaction rate. The general formula for the action of an enzyme is given by the following:

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(Equation 5.1)

where E = concentration of the enzyme
S & P = concentrations of substrate and product, respectivelye
ES & EP = concentration of enzyme-substrate complex and enzyme-product complex
k1-k4 = rate constants for each step.

From equation 5.1, the rates(velocities) of each reaction can be given by:

v1 = k1(E)(S); formation of enzyme-substrate complex
v2 = k2(ES); reformation of free enzyme and substrate
v3 = k3(ES) formation of product and free enzyme
v4 = k4(E)(P) reformation of enzyme-product complex

In steady state equilibrium, (v1-v2)=(v3-v4) and, if all product is either removed or does not recombine with the enzyme, then k4 = 0, and k1(E)(S)-k2(ES)=k3(ES)

This equation can then be rearranged to yield:

$$\frac{K2 + K3}{K1} = \frac{(E)(S)}{(ES)} \qquad \text{(Equation 5.2)}$$

where the left side of this equation can be given as a single constant, known as K_m, the rate constant, or the Michaelis constant. Note that the units for this constant will be those of concentration.

One of the important concepts of metabolism is that enzymes from differing sources may have the same function (i.e. the same substrate and product), but possess significantly different K_m values. Since biological function is as dependent on the rate of a reaction as it is on the direction of a reaction, it becomes necessary to measure the K_m value for any enzyme under study.

Enzymes act as catalysts because of their three dimensional protein structure. This structure is controlled by many factors, but is particularly sensitive to changes in pH, salts and temperature. Small changes in the temperature of a reaction can significantly alter the reaction rate, and extremely high temperatures can irreversibly alter both the three dimensional structure of the enzyme and its activity. It may even render the enzyme non-functional; that is, to denature the enzyme. Salts can also cause denaturation, but the effects of ammonium sulfate are usually reversible. Heavy metal salts, by contrast, usually irreversibly alter the structure of the protein, and thus their routine use as fixatives in histological work.

Active Sites

An enzyme works by binding to a given substrate in such a geometrical fashion that the substrate is able to undergo its inherent reaction at a more rapid rate. This type of reaction is commonly referred

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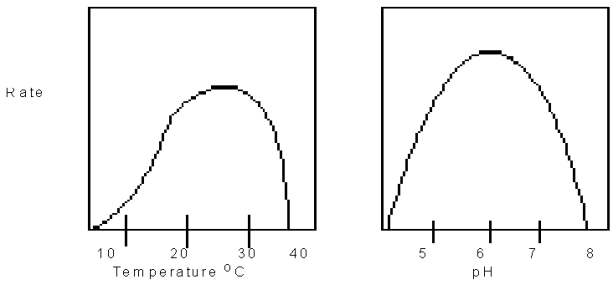
to as the lock and key mode for enzyme action. It implies that there is a particular part of the enzyme structure, the active site, which specifically binds sterically to a substrate. The enzyme does not actually react with the substrate but merely brings the substrate into the proper alignment or configuration for it to react spontaneously or in conjunction with another substance. Since a reaction proceeds normally by a random kinetic action of molecules bumping into each other, any time molecules are aligned, they will react faster. Thus, for any given enzyme there will be a best fit configuration to the protein in order to align the substrate and to facilitate the reaction. When the enzyme is in its ideal configuration, the reaction will proceed at its maximum rate, and the overall rate of activity will be dependent upon substrate concentration.

Figure 5.1 Hypothetical effect of temperature and pH.



Maximum reaction rate assumes that an optimal pH, salt environment and temperature have been established. Figure 5.1 demonstrates some typical effects of temperature and pH on the rate of an enzyme catalyzed reaction. Maximum rate further assumes the presence of any co-enzymes and/or cofactors that the enzyme requires. Co-enzymes are organic molecules which must bind to the protein portion of the enzyme in order to form the correct configuration for a reaction. Cofactors are inorganic molecules which do the same.

Now, if we measure the concentration of an enzyme via its rate of activity (i.e. the velocity of the catalyzed reaction), we must control the reaction for the effects of temperature, pH, salt concentration, co-enzymes, cofactors, and substrate concentration. Each of these parameters affects the rate of an enzyme reaction. Thus, each must be carefully controlled if we attempt to study the affects of changes in the enzyme itself. For example, alterations in the rate of a reaction are directly dependent upon the concentration of functional enzyme molecules only when the enzyme is the limiting factor in the reaction. There must be sufficient substrate to saturate all enzyme molecules in order for this criterion to be met. If the substrate concentration is lowered to the point where it becomes rate limiting, it is impossible to accurately measure the enzyme concentration, because there will be two variables at work.

Figure 5.2 Michaells-Menten plot of enzyme activity

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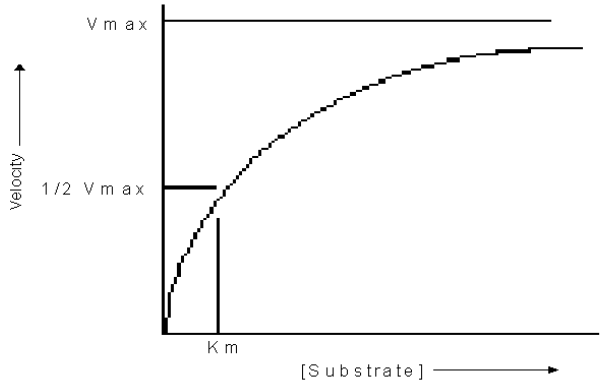
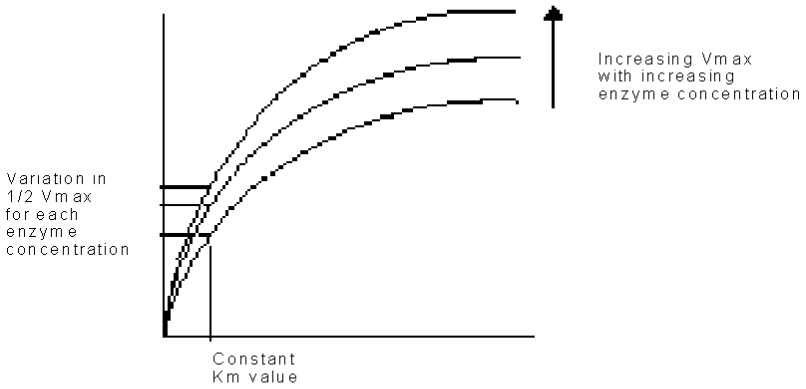


Figure 5.3 Effect of increasing enzyme concentration



The relationship between substrate concentration and enzyme concentration was mathematically established by pioneering work of two biochemists, L. Michaelis and M.L. Menten in 1913. In recognition of their work, the plots of enzyme activity vs. substrate concentration are known as Michaelis-Menten plots. These are relatively simple plots (Figure 5.2) in which the substrate concentration is on the x-axis, and the velocity of reaction is on the y-axis. The plot demonstrates that as the substrate increases, the velocity increases hyperbolically, and approaches a maximum rate known as Vmax. This is dependent upon saturation of the enzyme. At Vmax, all enzyme molecules are complexed with substrate, and thus any additional substrate added to the reaction has no effect on the rate of reaction.

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However, this situation becomes more complex: as you change the enzyme concentration, V_{max} will also change (Figure 5.3). Thus, V_{max} is not a constant value, but is constant only for a given enzyme concentration. Consequently, the value of V_{max} can not be used directly to infer enzyme concentration. It is dependent upon at least two variables, enzyme concentration and substrate concentration (assuming temperature, pH and cofactors have all been controlled). What Michaelis and Menten discovered was a simple means of solving the equations for two variables. If multiple plots of enzyme activity vs. substrate concentration are made with increasing enzyme concentration, the value of V_{max} continues to increase, but the substrate concentration which corresponds to 1/2 V_{max} remains constant. This concentration is the **Michaelis Constant** for an enzyme. As mentioned, it is designated as K_m and is operationally the concentration of substrate which will give exactly 1/2 V_{max} when reacted with an enzyme with maximum pH, temperature and cofactors.

According to the Michaelis-Menten equation:

$$V = \frac{V_{max}(S)}{K_m + (S)} \qquad \text{(Equation 5.3)}$$

This equation is derived from the formula for a hyperbola (c=xy) where $K_m = (S)(V_{max}/v-1)$

When $v=V_{max}/2$, $K_m = (S)(V_{max}/(V_{max}/2)-1) = (S)$ confirming that the units of this constant are those of concentration.

A Michaelis-Menten plot thus can give us an easy way to measure the rate constant for a given enzyme. An immediate difficulty is apparent, however, when Michaelis-Menten plots are used. V_{max} is an asymptote. Its value can only be certain if the reaction is run at an infinite concentration of substrate. Obviously, this is an impossible prospect in lab.

In 1934, two individuals, Lineweaver and Burke made a simple mathematical alteration in the process by plotting a double inverse of substrate concentration and reaction rate (velocity).

The Lineweaver/Burke equation is:

$$\frac{1}{v} = \frac{K_m + (S)}{V_{max}(S)} \qquad \text{(Equation 5.4)}$$

This equation fits the general form of a straight line, y=mx+b, where m is the slope of the line and b is the intercept (Figure 5.4). Thus, the Lineweaver/Burke Plot for an enzyme is more useful than Michaelis-Menten, since as velocity reaches infinity, $1/V_{max}$ approaches 0. Moreover, since the plot results in a straight line, the slope is equal to K_m/V_{max}, the y intercept equals $1/V_{max}$ (1/S=0). Projection of the line back through the x axis yields the value $-1/K_m$ (when 1/V=0). These values can easily be determined by using a linear regression plot and calculating the corresponding values for x=0 and y=0. The inverse of the intercept values will then yield V_{max} and K_m.

Figure 5.4 Lineweaver-Burke plot of enzyme activity

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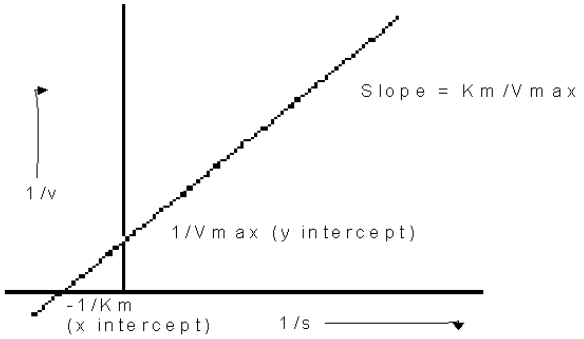

Remember that the point of all of these calculations is to determine the true activity and thus the concentration of the enzyme. If the reaction conditions are adjusted so that the substrate concentration is at K_m, then alterations in the rate of reaction are linear and due to alterations in enzyme concentration. Kinetic analysis is the only means of accurately determining the concentration of active enzyme.

Specific Activity

This brings us to a definition for enzyme activity. **specific activity** is defined in terms of enzyme units per mg enzyme protein. An enzyme unit is the amount of substrate converted to product per unit time under specific reaction conditions for pH and temperature.

As generally accepted, an enzyme unit is defined as that which catalyzes the transformation of 1 micromole of substrate per minute at 30° C and optimal chemical environment (pH and Substrate concentration). Specific Activity relates the enzyme units to the amount of protein in the sample.

While it is relatively easy to measure the protein content of a cell fraction, there may be a variable relationship between the protein content and a specific enzyme function. Remember that the initial extraction of an enzyme is accomplished by differential salt precipitation. Many proteins will precipitate together due to their solubility, but have no other common characteristics.

To determine both protein content and enzyme activity requires two different procedures. We can measure the amount of protein, or we can kinetically measure the enzyme activity. Combining the two will give us the specific activity.

Enzyme Inhibition

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Finally, before studying a specific enzyme, let's examine the problem of **enzyme inhibition**. Remember that enzymes function by sterically binding to a substrate. If a molecule interferes with that binding, it will hinder or inhibit the activity of the enzyme.

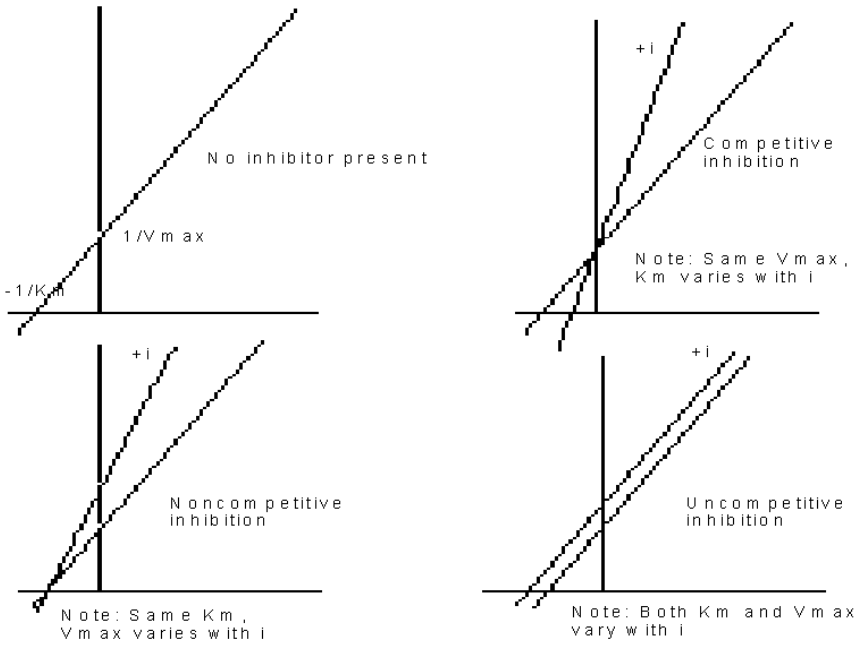
If the inhibitor molecule binds to the same active site as the substrate, then the reaction is known as competitive inhibition because the two molecules, substrate and inhibitor, compete for the same reaction site on the enzyme molecule. With this type of inhibition, V_{max} will not change because V_{max} is a function of all enzyme molecules uniting with substrate (thus having no effective competition). K_m, on the other hand, will alter with changes in the concentration of a competitive inhibitor, because it requires larger concentrations of substrate to overcome the direct competition of the inhibitor for the active site.

If, however, the inhibitor binds to a site on the enzyme other than the active site, then the inhibition is known as allosteric, or non-competitive inhibition. In this instance, the substrate and inhibitor bind to different parts of the enzyme molecule and thus are not in competition. An allosteric inhibitor alters the structure of the enzyme or physically blocks access to the active site. With non-competitive inhibition, V_{max} will change because, in effect, enzyme is being removed from the reaction. Its kinetic effects are equivalent to lowering the enzyme concentration. K_m will not change, however, since this value is constant regardless of the effective enzyme concentration.

Finally, there is a third class of inhibitor which can best be defined by its effect on V_{max} and K_m. Known as uncompetitive, it alters both of these values. It has effects on both the active site and allosteric sites.

The graphic representation of the three types of enzyme inhibition are show in Figure 5.5

Figure 5.5 Inhibitor effects on L-B plots



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Tyrosinase

This exercise involves the isolation (extraction) of the enzyme tyrosinase from potatoes and subsequent measurement of its activity. Tyrosinase is the common name for an enzyme that is formally termed Monophenol Monooxygenase and is listed as Enzyme #1.14.18.1 in the standard Enzyme Nomenclature. 1 It is also known as phenolase, monophenol oxidase and as cresolase. It is, functionally, an oxygen oxidoreductase enzyme.

This nomenclature points out another difficulty of working with enzymes. Their names are derived from the known activities. Enzymes isolated from different sources and measured for their catalytic activity with varying substrates can turn out to be the same protein. Thus, the enzyme tyrosinase, discovered in animal systems, was named for its action on the amino acid tyrosine, and specifically for its ability to form dopaquinone, an intermediate metabolite in the production of melanin.

The same enzyme isolated from plant materials had been examined for its ability to oxidize phenolic residues, and thus the names phenolase, monophenol oxidase and cresolase. Since it has been extensively studied in melanin production, we will continue to use the common name of tyrosinase.

The enzyme tyrosinase is fairly ubiquitous; that is, it is found in nearly all cells. In research, it has been purified from the fungus N. crassa 2 by freezing kilogram quantities of the fungal mycelia in liquid nitrogen, homogenizing the frozen tissue with a French Press, precipitating the proteins in ammonium sulfate, and purifying the enzyme chromatographically on Sephadex and Celite columns. A fairly complex undertaking.

Tyrosinase has been extracted from hamster melanomas by modifications of this technique and with the addition of acetone extractions as well as DEAE-cellulose chromatography and alumina treatments. 3 Tyrosinase has also been separated from many plant tissues utilizing a far simpler technique based principally on ammonium sulfate precipitation of proteins.

The catalytic action of this enzyme is the conversion of tyrosine + O_2 to yield dihydroxyphenylalanine (DOPA), which is then converted to dopaquinone + H_2O. Dopaquinone in turn can be readily converted to dopachrome, an orange to red pigment (found in human red hair), which can then be converted to the black/brown melanin pigments (found in virtually all human pigments).

Tyrosine + 1/2 O_2 -----> DOPA

2 DOPA + O_2 ----> 2 Dopaquinone + 2 H_2O

Dopaquinone ----> Leukodopachrome

Leukodopachrome + Dopaquinone ----> Dopachrome + DOPA

The enzyme catalyzes the first two of these reactions, namely the conversion of tyrosine and the conversion of DOPA. The formation of dopachrome from dopaquinone is spontaneous.

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We can now monitor the activity of the enzyme by analyzing the disappearance of tyrosine and/or DOPA as substrates, the appearance of leukodopachrome or dopachrome as products, or by monitoring the use of oxygen. Physiologists and chemists have long preferred the manometric determination of gaseous oxygen exchange, but far simpler is the determination of dopachrome, a natural pigment with an absorbance maximum at 475 nm. This absorbance allows us to use standard spectrophotometric analysis by analyzing the formation rate of dopachrome from the substrate DOPA.

The summary reaction for tyrosinase activity, as used in this exercise is

DOPA + 1/2 O_2 ----> Dopachrome

Exercise 5.1 - Extraction of Tyrosinase

LEVEL I

Materials

- Potatoes
- Paring Knife
- Blender
- 0.1 M NaF
- Rubber Gloves
- Saturated Ammonium Sulfate (4.1 M @25° C)
- Volumetric Cylinders (50 ml, 100 ml, 250 ml)
- Cheesecloth
- Beakers (100 ml, 250 ml)
- Chilled Centrifuge Tubes (30-50 ml)
- Refrigerated Centrifuge
- 0.1 M Citrate Buffer, pH 4.8
- Glass Stirring Rod

Procedure

1. Peel a small potato and cut into pieces about 1 inch square.
2. Add 100 grams of the potato to a blender, along with 100 ml of sodium fluoride (NaF). Homogenize for about one minute at high speed.

   Caution: Sodium Fluoride is a poison! Wear rubber gloves while handling, and wipe up any spills immediately.

3. Pour the homogenate (mixture) through several layers of cheesecloth and into a beaker.
4. Measure the volume of the homogenate and add an equal volume of saturated ammonium sulfate. That is, if the fluid volume of your homogenate is 150 ml, add 150 ml of ammonium sulfate. This will cause a floculent white precipitate to appear as many of the previously soluble potato proteins become insoluble. The enzyme tyrosinase is one of these proteins and thus will be found in the subsequent precipitate.

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5. Divide the ammonium sulfate-treated homogenate into chilled centrifuge tubes and centrifuge at 1,500 xg for 5 minutes @ 4° C.
6. Collect the centrifuge tubes, and carefully pour off and discard the fluid (supernatant). **Save the pellets.** Combine all of the pellets into a 100 ml beaker.
7. Add 60 ml. of citrate buffer, pH 4.8, to the pooled pellet and stir the contents well. Use a glass rod to break up the pellet. Continue to stir for 2 minutes while keeping the solution cool.
8. Again divide the solution into centrifuge tubes and recentrifuge at 300 xg for 5 minutes at 4° C.
9. Collect and save the supernatant. **This is your enzyme extract!** Place it in an erlenmeyer flask, label it as Enzyme Extract and place it in an ice bucket. The enzyme tyrosinase is insoluble in 50% ammonium sulfate, but is soluble in the citrate buffer. Keep this extract chilled for the duration of the laboratory. Tyrosinase is stable for about an hour under the conditions of this exercise. If not used within this period, you will need to extract more enzyme from a fresh potato.

Exercise 5.2 - Preparation of Standard Curve

LEVEL I

Materials

- 8 mM DOPA
- Enzyme Extract - From Exercise 5.1
- Test tubes
- 5 ml Pipette
- 0.1M Citrate Buffer, pH 4.8
- Spectrophotometer and Cuvettes

Procedure

1. Begin by preparing a standard solution of the orange colored dopachrome from L-DOPA. To 10 ml of 8 mM DOPA, add 0.5 ml of your enzyme extract 4 and allow the solution to sit for 15 minutes at room temperature. During this period, all of the DOPA will be converted to dopachrome, and your solution will now contain 8 mM dopachrome. Dopachrome is somewhat unstable in the presence of light and should be stored in an amber bottle or out of the light.
2. Prepare a 1:1 series of dilutions of the 8 mM Dopachrome to yield the concentrations in the following table: Add 3.0 ml of each indicated concentration to tubes #1-8.

Tube #	Final Concentration of Dopachrome (mM)
1	0
2	0.125
3	0.25
4	0.5
5	1.0
6	2.0

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7	4.0
8	8.0

3. With these dilutions, you have prepared tubes containing concentrations from 0 to 8 mM dopachrome (tubes 1-8). Tube 1 contains no dopachrome and is used for blanking the spectrophotometer.
4. The units of concentration are millimolar (mM). A 1.0 mM solution contains .001 moles per liter or .000001 moles per ml. Thus, with a volume of 3.0 ml, there are .000003 moles of dopachrome, or 3 micromoles. Correspondingly, tubes 2-8 contain 1 to 24 micromoles of dopachrome. For the remainder of this exercise, be sure to distinguish between concentration (mM) and total amount of substance present (micromoles).
5. Turn on your spectrophotometer and set a wavelength of 475. Use tube #1 from the above dilutions as a blank and adjust the spectrophotometer for 0 and 100% T. Read the absorbance (or read and convert transmittance) of each of the solutions in tubes 2-8 and complete the following table:

Tube	Concentration of Dopachrome (mM)	Absorbance	A/C
#1	0	0	----
#2	0.125		
#3	0.25		
#4	0.5		
#5	1.0		
#6	2.0		
#7	4.5		
#8	8.0		

6. Calculate the values for the last column of the table. This column represents the simplest calculation of the extinction coefficient for dopachrome absorbance. Average the values in this column and enter the number at the bottom of the column. This is the average extinction coefficient and can be used in subsequent determinations of dopachrome concentrations according to the Beer-Lambert law.

   You can more accurately determine the extinction coefficient by performing a linear regression analysis of your data, and computing the slope and y intercept. The slope of the linear regression will represent the extinction coefficient for your sample.

7. Plot a scattergram of the absorbance value against the concentration of dopachrome. The known concentration of dopachrome should be the x axis, while absorbance should be the y axis.
8. Plot the computed slope and intercept of the linear regression as a straight line overlaying your scattergram. The equation for a straight line is y = mx + b, where m is the slope and b the intercept. [5]

Notes

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Since tyrosinase catalyzes the conversion of L-DOPA to dopachrome, this exercise measures the conversion of colorless DOPA to the dark orange dopachrome. Substrate and product are in a 1:1 ratio for this reaction, thus the amount of product formed equals the amount of substrate used. The optical density of dopachrome @475 nm is directly proportional to the intensity of orange color formation in solution (Beer-Lambert Law).

Exercise 5.3 - Enzyme Concentration

LEVEL I

Materials

- Enzyme Extract
- 0.1 M Citrate Buffer, pH 4.8
- 10 ml Pipette
- 8 mM DOPA
- Spectrophotometer and Cuvettes
- Ice Bath

Procedure

1. To determine the kinetic effects of the enzyme reaction, first determine an appropriate dilution of your enzyme extract. This will give a rate of reaction of 5-10 micromoles of DOPA converted per minute. Prepare a serial dilution of your enzyme extract. Place 9.0 ml of citrate buffer into each of three test-tubes. Label the tubes 1/10, 1/100 and 1/1000.
2. Pipette 1.0 ml of your enzyme extract into the first of these tubes (the one labeled 1/10) and mix by inversion.
3. Pipette 1.0 ml of the 1/10 dilution into the second tube (labeled as 1/100) and mix by inversion.
4. Pipette 1.0 ml of the 1/100 dilution into the third tube (labeled as 1/1000) and mix by inversion.
5. Place all of the dilutions in the ice bath until ready to use.
6. If not already done, turn on a spectrophotometer, adjust to 475 nm and blank with a tube containing 2.5 ml of citrate buffer and 0.5 ml of enzyme extract.
7. Add 2.5 ml of 8 mM DOPA to each of 4 cuvettes or testtubes. Note that each tube contains .0025 X .008 moles or 20 micromoles of DOPA.
8. Add 0.5 ml of undiluted enzyme extract to one of the tubes containing the 8 mM DOPA. Mix by inversion, place into the spectrophotometer and immediately begin timing the reaction. Carefully measure the time required for the conversion of 8 micromoles of DOPA. Note that since the cuvette will contain a volume of 3.0 ml, the concentration when 8 micromoles are converted will be 8/3.0 or 2.67 mM dopachrome. Use the data from the standard curve (Exercise 5.2) to determine the absorbance equal to 2.67 mM dopachrome. This absorbance value will be the end point for the reaction.

The Absorbance equal to 3.33 mM dopachrome (from Exercise 5.2) = _____

9. As the reaction takes place within the spectrophotometer, the absorbance will increase as dopachrome is formed. When the absorbance reaches the value above, note the elapsed time

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from the mixing of the enzyme extract with the 10 mM DOPA. Express the time as a decimal rather than minutes, seconds. The time should be between three and five minutes. If the end point is reached before three minutes, repeat Step 8, but using the next dilution of enzyme (i.e. the 1/10 after the undiluted, the 1/100 after the 1/10 and the 1/1000 after the 1/100).

The rate of activity = _____ micromoles/minute/0.5 ml of diluted extract. The dilution factor (inverse of dilution, 1,10,100 or 1000) is _____. The activity of the undiluted enzyme is _____ micromoles/minute/0.5 ml or _____ micromoles/minute/1.0 ml of extract.

10. For the enzyme dilution which reaches the end point between 3 and 5 minutes, calculate the velocity of reaction. Divide the amount of product formed (10 micromoles) by the time required to reach the end point.

Exercise 5.4 - Effects of pH

LEVEL I

Materials

- 8 mM DOPA in citrate buffer adjusted to
  pH values of 3.6, 4.2, 4.8, 5.4, 6.0, 6.6, 7.2, 7.8
- Enzyme Extract
- Spectrophotometer and Cuvettes
- Stopwatch

Procedure

1. Set up a series of test tubes each containing 2.5 ml of 8 mM DOPA, but adjusted to the following pH values: 3.6, 4.2, 4.8, 5.4, 6.0, 6.6, 7.2, and 7.8
2. Begin with the tube containing DOPA @ pH 3.6, add 0.5 ml of the diluted enzyme extract which will convert 10 micromoles of DOPA in 3-5 minutes as determined in Exercise 5.3. Start timing the reaction, mix by inversion and insert into the spectrophotometer. Note the time for conversion of 10 micromoles of DOPA.
3. Repeat Step 2 for each of the indicated pH values. Complete the following table:

pH	Time(Minutes)	Micromoles of Dopachrome	Velocity (Micromoles/Minute)
3.6		10	

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4.2		10	
4.8		10	
5.4		10	
6.0		10	
6.6		10	
7.2		10	
7.8		10	

4. Plot pH (x-axis) versus Reaction Velocity (y-axis)

Exercise 5.5 - Effects of Temperature

LEVEL I

Materials

- Enzyme Extract
- 8 mM DOPA pH 6.6
- Incubators or Water baths adjusted to 10, 15, 20, 25, 30, 35 and 40° C [6]
- Spectrophotometer and Cuvettes
- Stopwatch

Procedure

1. Set up a series of test tubes each containing 2.5 ml of 8 mM DOPA buffered to a pH = 6.6. Place one tube in an ice bath or incubator adjusted to the following temperature; 10, 15, 20, 25, 30, 35 and 40° C
2. Add 0.5 ml of an appropriately diluted enzyme extract (to yield 10 micromoles dopachrome in 3-5 minutes) to each of a second series of tubes. Place one each in the corresponding temperature baths. Allow all of the tubes to temperature equilibrate for 5 minutes. **Do not mix the tubes.**
3. Beginning with the 10° C tube, and with the spectrophotometer adjusted to 475 nm and properly blanked, pour the enzyme (0.5 ml @ 10° C) into the tube containing the DOPA and begin timing the reaction. Mix thoroughly. Note the time to reach the end point equivalent to the conversion of 10 micromoles of substrate.
4. Repeat Step 3 for each of the listed temperatures, complete the following and plot the data.

Temperature (° C)	Time (Minutes)	Micromoles of Dopachrome	Velocity (Micromoles/Minute)

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10		10	
15		10	
20		10	
25		10	
30		10	
35		10	
40		10	

Exercise 5.6 - Computer Simulation

LEVEL I

You will note that for the determination of pH and temperature effects, we established conditions that scanned a wide range of the variables. To refine these procedures, we would add more values, for example, temperatures from 30- 40° C at steps of 1.0° C or 36-37° C with a step of 0.1° C. This is time consuming.

To establish the principle, however, let's shift to computer simulation of enzyme activity. Computer simulations will also assist in the rapid accumulation of data for kinetic analysis.

There are several excellent commercial programs available for enzyme kinetic analysis, and use of these must be left to the descretion of the instructor. The author has had excellent results with a rather old BASIC program, ENZKIN, which is still available from Conduit. 7

This is a simple, straight-forward and inexpensive program which is available for Apple Computers and for main frames. It can readily be converted to run on other machines with some simple source code changes. Unfortunately, Conduit may not continue supplying this program in the future.

If you do not wish to convert programs, or if you prefer a more sophisticated program is desired, the author recommends ENZPACK Ver. 2.0. 8 In addition to simulation of enzyme kinetics, this program allows entry of data with subsequent graphing and analysis. It also allows for high order kinetic analysis in addition to Lineweaver-Burk plots.

Exercise 5.7 - Kinetic analysis

LEVEL II

Materials

- 8 mM DOPA pH 6.6
- Enzyme Extract, diluted to yield 10 micomoles of dopachrome in 3-5 minutes (Exercise 5.2)

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- Spectrophotometer and Cuvettes
- Stopwatch

Procedure

1. Prepare a reaction blank in a clean cuvette to contain 2.5 ml of citrate buffer and 0.5 ml of enzyme extract. Use this blank to adjust your spectrophotometer for 100% transmittance. Remove the blank and save.
2. Add 2.5 ml of 8 mM DOPA, pH 6.6 to a clean cuvette.
3. Add 0.5 ml of appropriately diluted enzyme extract. Shake well and immediately insert the tube into the spectrophotometer. Record the absorbance or transmittance as quickly as possible. Designate this reading as time 0.
4. At 30 second intervals read and record the transmittance until a transmittance value of 10% (Absorbance = 1.0) is reached. Complete the following table:

Time (Minutes)	Absorbance	Concentration of Dopachrome(mM)	Micromoles of Dopachrome
0.5			
1.0			
2.0			
2.5			
3.0			
3.5			
4.0			
4.5			
5.0			

5. Plot time in minutes (x-axis) versus the amount of dopachrome formed (y-axis).

Exercise 5.8 - Determination of Km and Vmax

LEVEL II

Materials

- Enzyme Extract
- 8 mM L-DOPA adjusted to pH 6.6
- Spectrophotometer and Cuvettes
- Stopwatch

Procedure

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1. Dilute the DOPA standard (8 mM) to obtain each of the following concentrations of L-DOPA: 0.5 mM, 1 mM, 2 mM 4 mM, and 8 mM.
2. Repeat Exercise 5.7 for each of the substrate concentrations listed, substituting the change in concentration where appropriate.
3. Plot each set of data and from the data calculate the time required to convert 10 micromoles of DOPA to dopachrome. Compute the velocity of enzyme reaction for each substrate concentration. Fill in the following table:9

Substrate (DOPA) Concentration (mM)	**Velocity Micromoles/Minute**	**1/s**	**1/v**
0.5		2.00	
1.0		1.00	
2.0		0.50	
4.0		0.25	
8.0		0.125	

4. Plot the rate of DOPA conversion (v) against substrate concentration in the appropriate place below. This is a Michaelis-Menten plot.
5. Plot a double reciprocal of the values plotted in step 4; that is, 1/s versus 1/v. This is a Lineweaver-Burke plot.
6. Perform a linear regression analysis on the second plot and compute the slope and both y and x intercepts.

Note that the x intercept is -1/K_m, the negative inverse of which is the Michaelis-Menten Constant. The y intercept is 1/V_{max} and the slope equals Km/V_{max}.

Exercise 5.9 - Addition of Enzyme Inhibitors

LEVEL II

Materials

- Enzyme Extract
- 8 mM L-DOPA
- 8 mM Benzoic Acid
- 8 mM KCN
- 0.1 M Citrate Buffer, pH 6.6

Procedure

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1. Tyrosinase is inhibited by compounds that complex with copper, as well as by benzoic acid and cyanide. To determine the inhibitory effects of benzoic acid and cyanide, set up a series of tubes as indicated.
2. Using one tube at a time, add 0.5 ml of the enzyme dilution previously calculated to yield 10 micromoles of dopachrome in 2-3 minutes. For each tube, measure the time required to convert 10 micromoles of DOPA to dopachrome. Enter those times in the table below. Compute the reaction velocity for each substrate concentration:

Tube #	**Time (Minutes)**	**Final [DOPA] nM**	**Velocity Micromoles/Minute**
1 Benzoic Acid		6.67	
2 Inhibited		6.00	
3 Series		5.33	
4		4.67	
5		4.00	
6		3.33	
7		2.67	
8		2.00	
9		1.33	
10		0.67	
11		0	
12 KCN		6.00	
13 Inhibited		5.33	
14 SERIES		4.67	
15		4.00	
16		3.33	

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17		2.67	
18		2.00	
19		1.33	
20		0.67	
21		0	

3. Calculate the values for 1/s and 1/v for each of the corresponding s and v in the table below. Plot 1/v vs 1/s for the presence of benzoic acid and a second plot for the presence of KCN. Compute the values of V_{max} and K_m for the presence of each inhibitor. Determine whether these inhibitors are competitive, non-competitive or uncompetitive.

Benzoic Acid Inhibition			
Tube #	**8 mM DOPA**	**8 mM Benzoic Acid**	**Buffer**
1	2.0	0.5	0
2	1.8	0.5	0.2
3	1.6	0.5	0.4
4	1.4	0.5	0.6
5	1.2	0.5	0.8
6	1.0	0.5	1.0
7	0.8	0.5	1.2
8	0.6	0.5	1.4
9	0.4	0.5	1.6
10	0.2	0.5	1.8
11	0	0.5	2.0
KCN Inhibition			

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Tube #	8 mM DOPA	8 mM KCN	Buffer
12	1.8	0.5	0.2
13	1.6	0.5	0.4
14	1.4	0.5	0.6
15	1.2	0.5	0.8
16	1.0	0.5	1.0
17	0.8	0.5	1.2
18	0.6	0.5	1.4
19	0.4	0.5	1.6
20	0.2	0.5	1.8
21	0	0.5	2.0

Exercise 5.10 - Protein Concentration/Enzyme Activity

LEVEL II

Materials

- Commercially pure tyrosinase
- UV Spectrophotometer
  or Materials for Lowry or Bradford Protein determination
- L-DOPA
- 0.1 M Citrate buffer, pH 6.6

Procedure

1. Prepare a solution of 0.7 micrograms of commercially pure tyrosinase diluted to 4 ml. with 0.1 M Citrate Buffer, pH 6.6.
2. Measure the OD_{280} of your sample and prepare a dilution of your enzyme extract to a final concentration of 0.7 micrograms in 4 ml of citrate buffer.
3. Place both enzyme samples in a water bath @ 30° C for 5 minutes to temperature equilibrate.
4. Turn on the spectrophotometer, set the wavelength to 475 nm and blank the instrument using citrate buffer as the blank.
5. Select the commercial preparation and add exactly 1.0 ml of L-DOPA (4 mg/ml in citrate buffer) and

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immediately read the absorbance at 475 nm.

6. Replace the tube in the water bath and wait exactly 5 minutes. Read the OD_{475} immediately.
7. The molar absorbance coefficient for dopachrome is 3.7 x 10^4. Use this value to compute the specific activity of the commercial enzyme preparation. Check this activity against that listed with the enzyme preparation.
8. Repeat steps 5 and 6 with your extracted enzyme preparation. Compute the specific activity (enzyme units of activity/ mg protein) of your enzyme preparation. *Enzyme Unit: The absorbance reading under the conditions specified in this exercise is proportional to the enzyme concentration, where 1 unit of enzyme activity yield a 0.81 OD change in readings.*

Notes

The protein content can be measured by the Lowry or Biuret procedures found in the Appendix G, or more simply by a single spectrophotometric measure of the absorbance of the sample at 280 nm. Without going into mathematical detail, a 1% pure solution of tyrosinase has an OD_{280} equal to 15.6/cm. The Beer-Lambert law can thus be used to determine protein content in a non-destructive manner.

Exercise 5.11 - Advanced Analysis of Enzyme Parameters

LEVEL III

In the extraction procedure for tyrosinase, only one precipitation was employed utilizing ammonium sulfate. This procedure yields a rapid means for obtaining the enzyme, but results in increased contamination and lower yield. The following improved procedure uses a serial increase in ammonium sulfate.

After blending the potato, add 1/2 volume of ammonium sulfate to yield a 33% solution as opposed to 50%. Collect the precipitate and label it as P1. Add another 1/2 volume of ammonium sulfate to raise the concentration to 50% and collect and label the resulting precipitate as P2. Finally add a third 1/2 volume to raise the concentration to 67% and collect and label the precipitate as P3. Use each of these to measure the specific activity as well as the total activity in each fraction. The step-wise addition of ammonium sulfate will increase the specific activity. If pure enzyme is desired, the steps can be made even smaller, going from 30% to 70% in 10% increments.

If you wish further purification, then subject the resulting precipitated protein to electrophoretic separation and analyze the resulting bands for their tyrosinase activity. While these procedures can yield pure enzyme fractions, they are more pertinent to biochemical studies.

A more interesting analysis can be made by comparing tyrosinase extracted from differing biological materials (mushrooms, bananas, melanocytes) for the values of Km and for their optimum pH and temperature. Based on those differences, might it be possible to predict the darkening of fruit under varying conditions of pH and temperature?

Finally, the enzyme can be studied in a structure linked mode by combining aspects of cell organelle isolation (Chapter Three) to the activity of the enzyme. The presence (or absence) of

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tyrosinase can be monitored within the organelles of various cells. Melanocytes would be an excellent source of cellular tyrosinase. You could then correlate the activity of tyrosinase with the production of the pigment melanin.

Endnotes

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1. Numerical order or enzymes as classified by the International Union of Biochemistry.
2. N.H. Horowitz, M. Gling and G. Horn. Tyrosinase (*Neurospora crassa*) in Methods in Enzymology, Vol. XVII. (H. Tabor and C.W. Tabor eds.) p. 615- 620. 1970.
3. S.H. Pomerantz and J.P. Li, Tyrosinase (Hamster Melanoma) in Methods in Enzymology, Vol XII, p 620-626.
4. If the extraction of tyrosinase is to be skipped, a commercial preparation of 0.7 micrograms of tyrosinase (4,000 units/mg) in 4 ml of citrate buffer may be substituted.
5. This can be easily accomplished with many standard "scientific" calculators, most computer spreadsheets, or with the BASIC program listed in Appendix D.
6. The exercise is easier to perform if a temperature controlled spectrophotometer is available, since the reaction rate can be monitored at the proper temperature. Lacking this piece of equipment, however, the reaction can be placed in an ice bath or incubator and monitored every 30 seconds.
7. Conduit, University of Iowa, Oakdale Campus, Iowa City, Iowa 52242.
8. BIOSOFT, PO Box 580, Milltown, NJ 08850.
9. Note that the table lists the concentrations as those of the initial substrate. This is not technically correct since the addition of 0.5 ml of enzyme will dilute the substrate. If Exercise 5.9 is to be performed, the values of 1/s will need to be adjusted accordingly.

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Chapter 6: Membranes

Introduction

Although not all researchers agree on the fundamental nature of membranes, all agree that membranes take on properties fundamental to the very existence of life. Following the theory of Albert Szent-Gyorgyi, they believe that the understanding of life can be achieved through an understanding of the mechanisms for electron flow through basic organic systems (photosynthesis and respiration) and that these systems in turn exist only through compartmentalization within a cell. Without it, there would be no organelles, and indeed, no cell.

While membranes can be studied within living cells, membrane composition requires isolation and subfractionation of the membrane components. Once isolated, the membranes can be solubilized through the use of detergents and analyzed for proteins, lipids and carbohydrates. The study of biomembranes can be done with techniques requiring minimal equipment, such as osmotic shrinking or swelling of erythrocytes. Some techniques can be so complex as to require a complete organic and physical chemistry lab. The labs in this exercise can be completed with the equipment in the average college biochemistry lab.

The earliest investigations of membranes were those concerned with chemical analysis and were based on the observation that lipid solvents readily permeated cells. Membranes were known to contain protein, and from these early studies, it was concluded that lipids were also a significant factor in membrane composition. The specific nature of the proteins and lipids has been the subject of much research since these early days. As an example of the protein and lipid composition of membranes, as well as the percentage of some other substances, Table 6.1 presents the composition of myelin membranes from the central nervous system and the brain.

Table 6.1 Composition of rat CNS myelin and brain

Substance	Myelin	Whole Brain
Total protein	29.5	56.9
Total lipid	70.5	37.0
Cholesterol	27.3	23.0
Total galactolipid	31.5	21.3
Cerebroside	23.7	14.6
Sulfatide	7.1	4.8
Total phospholipid	44.0	57.6
Ethanolamine phosphatides	16.7	19.8
Choline phosphatides	11.3	22.0

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Serine phosphatides	7.0	7.2
Inositol phosphatides	1.2	2.4
Sphingomyelin	3.2	3.8
Plasmalogens	14.1	11.6
Total protein and lipid as percent dry weight, all others percent total lipid.		

Myelin is one of the most often studied biomembranes. It is readily available in quantity, and has a relatively simple composition when compared to membranes from other cells. There are three major types of protein found associated with myelin, basic protein, Folch-Lees proteolipid, and Wolfgram protein. There is a high percentage of lipid composition and the lipids are reasonably uncomplicated.

Of course, this is a simple view of myelin based on early chemical extractions. Anatomists have long recognized the distinction between myelin from the central nervous system (CNS) and that from the peripheral nervous system (PNS). CNS myelin is formed by oligodendrocytes, while PNS myelin is formed by the Schwann Cells. The PNS system is the most often illustrated because of the ease of identification of the laminar membranes of the system. Biochemical analysis of myelin further indicates that there may be subtle differences even within the broad categories of myelin, with, for example, clear distinction between that found in the brain and the spinal cord of the CNS, additional distinctions among species.

Often the chemical composition is of interest by itself, but more intriguing are the dynamic characteristics of membranes, (such as semi-permeability) and functions (such as active transport and facilitated transport). To study these properties, a relatively new approach has been developed: the synthesis of artifical membranes in the form of lipid bilayers (also referred to as bilayer lipid membranes or BLM's). The lipid bilayers are composed of natural or synthetic lipids that are artificially held between two aqueous environments. Table 6.2 presents a list of the major lipids used for lipid bilayer formation, while Table 6.3 indicates some of the membranes these systems have modeled. Artificial membrane research has been significantly advanced by the addition of membrane components extracted from biological systems. The components have come from brain extracts, chloroplast or mitochondrial membranes, and have been augmented with oxidized cholesterol and any number of surfactants. Researchers have been able to create fairly complex artificial membranes and which can mimic nearly all of the qualities of a cell membrane. They have formed the lipid membranes as sheets spread across tiny apertures, or as small droplets within an aqueous environment. Sheets are more relevant to membrane permeability studies, while droplets (known as liposomes) are useful for analyzing cell fusions and membrane flow.

Table 6.2 Lipids used for bilayer formation

Extracts	Lipids	Miscellaneous
Bovine Brain	Oxidized cholesterol	Chlorophylls
Yeast	7-dehydrocholesterol	Xanthophylls

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Sheep erythrocyte	Phosphatidyl ethanolamine	Retinol
E. coli	Phosphatidyl inositol	trans and cis retinal
Rat liver mitochondria	Phosphatidyl serine	B-carotene
Chlorella	Sphingomyelin	Phosphatidylglycerol
Spinach chloroplast	1,2-dipalmitoyl-glycerol-3-phosphoryl choline	Diglucosyldiglyceride
S. fecalis spheroplast	Egg and synthetic lecithins	

Table 6.3 Experimental models for biological membranes

Model	Components
Plasma membrane	Brain lipids Phospholipid and cholesterol Erythrocyte extract E. coli extract
Nerve cell membrane	Brain lipids Oxidized cholesterol plus dodecyl acid phosphate
Mitochondrial membrane	Phospholipid plus cholesterol Oxidized cholesterol plus dodecyl acid phosphate
Thylakoid membrane	Chlorophylls plus phospholipid Chloroplast extract
Visual receptor membrane	Carotenoid pigments plus phospholipid plus oxidized cholesterol

Yet another approach to membrane analysis has been the use of markers or probes. The work utilizes either enzymes or immunofluorescenct reagents. More recently, fluorescent dyes have been covalently attached to specific membrane proteins, which can in turn be micro-injected into cells and their paths through the cell and monitored by computer aided video systems. With this new advancement, quantitative as well as qualitative analysis of membrane flow in a dynamic living cell is possible.

These chemical studies augment the abundant data available from the work of virologists on such phenomena as capping, and membrane flow related to viral reproduction. They are also supplemented by studies of vessicle/vacuole dynamics during cell endocytosis.

Finally, physiologists have studied ion flux across membranes. No view of membranes would be complete without discussion of osmosis, diffusion, active transport and facilitated diffusion. These processes of flux are mathematically defined by the movement of ions across the membrane. While virtually every type of cell has been studied, the major work has involved the erythrocyte and various components of nerve and muscle function.

Through all of these approaches, a coherent theory for membrane structure is emerging. Most membrane analysts believe that essentially membranes are lipids in fluid suspension between two

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aqueous phases (inside and outside the cell), while the proteins are then attached to this lipid bilayer. Some researchers, however, feel that the protein matrix of the membrane is set and gives substance to the membrane. Lipids are then attached to a protein substrate due to their hydrophobic or hydrophyllic tendencies. This latter view would account for the more structured nature of polar cellular membranes, which do not appear to behave strictly as fluids.

Exercise 6.1 - Lipid Solubility of Membranes

LEVEL I

Materials

- Fresh beets
- Solutions of the following alcohols:
  - 22 M Methanol
  - 8.5 M Ethanol
  - 3.0 M n-Propanol
  - 1.1 M n-Butanol
  - 0.38 M Amyl alcohol (Optional)
- Razor blades
- Depression slides
- Stopwatch
- Microscope

Procedure [1]

1. Beet cells contain a high concentration of the red pigment anthocyanin. When exposed to a compound which dissolves the cell membranes, the anthocyanin will leak out of the cells and cause a red color to occur in the surrounding media.
   Cut thin slices of a beet so that they can be placed on a microscope depression slide and viewed with the lowest power (4X).
2. While watching the edge of the sliced beet, add approximately 1.0 ml of each of the above alcohols to the slide, until the beet section is submerged. Be careful not to allow the alcohol to flow off the slide.

   Iso-amyl alcohol has a strong, obnoxious odor and the fumes are somewhat irritating. Adequate ventilation is required.

3. Immediately begin to time the **dissolution** of the beet cell membranes. Mark the time when a red color is first observed in the surrounding alcohol solution.
4. Repeat the entire series for 1/2 and 1/4 dilutions of each of the alcohols.
5. For each dilution of each alcohol, calculate a penetration coefficient by dividing the time of pigment appearance by the molar concentration of the alcohol. In the space given on page 137, plot this penetration coefficient against the relative miscibility of the alcohol (known as the partitition or distribution coefficient).

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Alcohol	Formula	Molecular Weight	Partition coefficient
Methanol	CH_3OH	32.04	0.01
Ethanol	C_2H_5OH	46.07	0.03
n-Propanol	C_3H_7OH	60.09	0.13
n-Butanol	C_4H_9OH	74.12	0.58
n-Amyl alcohol	$C_5H_{11}OH$	88.15	2.00

Exercise 6.2 - Osmosis

LEVEL I

Materials

- Elodea, guard cells from plant leaves or animal blood
- Solutions ranging from 0.05 M to 0.50 M each of sucrose, sodium chloride, potassium chloride and calcium nitrate
- Razor blades
- Depression slides
- Microscope
- Osmometer (Optional)

Procedure

1. Place 0.5 ml of 0.50 M NaCl into the center of a depression slide and add either a small piece of an Elodea leaf, the stripped lower epidermal layer of a leaf containing guard cells, or a small drop of blood.
2. Place the slide on the microscope and observe the cells for swelling or shrinking.
   Swelling is difficult to determine with Elodea since the cell wall inhibits swelling. Cell shrinking can be observed as a pulling away of the cell membrane from the cell wall. In guard cells, the swelling will open the stomates, while shrinking will cause the stomates to close. Red blood cells react quickly to changes in the environmental salt concentrations and will shrink or swell. Shrinking is observed as a wrinkling or crenulation of the cell, and swelling may proceed to the point where the cells burst (plasmolysis).
3. Repeat steps 1 and 2 sequentially with 0.4, 0.3, 0.2, 0.1 and 0.05 M solutions of NaCl. Note which solution induces cellular shrinking or swelling.
   One solution will result in neither shrinking nor swelling. This is known as the isotonic solution. Those causing the cell to shrink are known as hypertonic, and those causing swelling are hypotonic.
4. Repeat steps 1-3 with each of the remaining salts and with sucrose. Note which concentration of each is the equivalent of the isotonic NaCl.
   Tonic refers to the NaCl equivalence of a solution. It is more correct for other salts and especially for organic non- electrolytes to use the terms isosmotic rather than isotonic.

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5. Based on the molarity of the salt solutions, calculate the osmolarity of each solution and compare the osmolarity of each solution that is isosmotic to the cells under study. Multiplying the osmolarity by 22.4 will yield the osmotic pressure in atmospheres.

Optional

Use an osmometer to determine the precise osmolarity of each solution. Osmometers measure the freezing point depression, which in turn can be related to an equivalent solution of NaCl. If the osmometer is available, follow the manufactuer's instructions for its use.

Exercise 6.3 - Pinocytosis

LEVEL I

Materials

- Actively growing culture *Amoeba proteus* 2
- 0.001 M Alcian blue
- 0.01 M Sodium azide
- Petroleum jelly
- Slides, coverslips
- Microscope

Procedure

1. Form a small ring of petroleum jelly onto a microscope slide.
2. Add a small drop of Amoeba suspension, and add about 10 µl of alcian blue to the slide. Note the time. Immediately place a coverslip onto the slide and gently press to seal the amoeba within the petroleum jelly ring.
3. Observe the Amoeba with 10 X magnification of the microscope. 3
4. Record the time for each of the following events:

    a. Surface staining
    b. Rounding up of the cell
    c. Formation of rosette (crinkling of cell)
    d. Channeling (flattened pseudopodia and invagination of membranes)
    e. Pinosome formation (small vessicles of dye pinched off from invaginated membranes).

5. Prepare another slide with a jelly ring, but add 10 µl of sodium azide along with the Amoeba. Allow the Amoeba to remain in the azide solution for about 5 minutes and then add the alcian blue and a coverslip. Repeat steps 3 and 4.
6. Compare the times for each event of step 4 for the control and for those Amoeba treated with the azide.

Notes

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Membranes normally do not allow larger molecules (such as dyes) to enter a cell through simple diffusion. If a dye is presented to an amoeba, however, the dye will be incorporated as part of what is referred to as receptor mediated endocytosis. In more general terms, the cell will drink or eat the dye, i.e. pinocytosis (cell drinking) or phagocytosis (cell eating). The dye will enter into internal vacuoles, known as food vacuoles.

With some dyes, the cell will actively transport the dye back out of the cell. It requires energy for this dye exclusion, energy derived from metabolism. Energy is only produced in living cells, and the phenomenon can be monitored in what is known as the dye exclusion test. The dye exclusion test becomes a way to monitor cell viability (Chapter 12).

For now, however, it implies that we can monitor the health of an amoeba by timing the movement of dye, and we can observe alterations in membrane function by alterations in the process of dye movement. A healthy, functioning cell will not stain and will have a minimum number of dyed vacuoles. Its membrane will remain intact and there will be minimum interruption of its ligand-mediated endocytotic processes. A damaged cell, however, will undergo physiological and morphological changes as the membrane receptor sites become irreversibly bound to dye. As it loses its ability to excrete the dye, it will become stained, round up and ultimately die.

Exercise 6.4 - Computer Simulation of Membrane Function

LEVEL I

Materials

- CELLM program found in Appendix D
- Computer capable of running BASIC

Procedure

1. Turn on the computer, boot with an appropriate DOS, and load the program CELLM.

   The program asks you to make decisions about the amount of water, sugar, ions and wastes that are within an *E. Coli* cell. Changes in the cell due to osmosis and diffusion take place automatically. Energy is required for the movement of wastes and for other functions (such as preparation for reproduction). As you utilize the sugar for energy, wastes will build automatically. To dispose of waste requires 4 molecules of sugar.

   From the time you begin the program, the cell will burn sugar for energy. Diffusion and osmotic potentials go into effect. If you successfully manipulate the parameters, the cell will divide. Otherwise, your cell dies.

2. Your initial concentrations of materials are as follows:

Water	67% or 6700 molecules

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Sugar	30 molecules
Sodium	0 molecules
Potassium	53 molecules
Waste	0

3. Your cell is then placed into a solution containing:

Water	87% or 8700 molecules
Sugar	10% or 1000 molecules
Sodium	trace
Potassium	trace
Waste	0

4. You must adjust the five parameters of the cell, while maintaining viability. Your safe concentrations are:

Water	6000-8000 molecules
Sugar	10-35 molecules
Sodium	0-13 molecules
Potassium	40-53 molecules
Waste	0-10 molecules

5. While you play the role of a membrane, attempt to understand the relationship of the various components to each other.
6. Submit a single page report on the game, detailing the components, and how they interact to make the cell divide.

Exercise 6.5 - Extraction of Brain Lipids

LEVEL II

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Materials

- Calf brain 4
- Chloroform
- Methanol
- 0.1 N HCl
- Blender or tissue homogenizer
- Refrigerated preparative centrifuge
- Large bucket rotor
- Freeze dryer
- Buchner funnel and filter paper (with cold trap)

Procedure

1. Homogenize 25 g of white matter from calf brain, in 500 ml of chloroform:methanol mixture (2:1, v/v).

   Do not use an ordinary blender. The solvents will dissolve the gasket and may burst into flames!

2. Centrifuge the mixture in a large scale rotor @ 10,000 xg for five minutes at 4 ° C to clarify the solution.
3. Add 20 ml. of distilled water per 100 ml of solution from step 2 and shake to form an emulsion.
4. Lyophilize to dryness.
5. Dissolve the residue in 50 ml of chloroform and filter through three layers of filter paper in a buchner funnel. Use a water vacuum system and a cold trap to prevent chloroform vapors from entering a pump and causing a fire.
6. Add 1.2 volumes of methanol.
7. Add 1.5 volumes of 0.1 N HCl and shake vigorously.
8. Centrifuge and collect the lower phase. Discard supernatant and interphase material.
9. Filter the lower phase three times with fresh filter paper.
10. Add 1/3 volume of methanol to the filtrate and store in 1.0 ml lots in a freezer. The extract will remain stable for approximately 2 months.

Exercise 6.6 - Formation of Lipid Bilayer

LEVEL II

Materials

- Lipids extracted per Exercise 6.5
- α-Tocopherol
- Cholesterol
- 10 ml Teflon cup and larger polystyrene beaker
- Sable paintbrush
- 0.1 M NaCl
- Interference scale

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Procedure

1. Form a working lipid bilayer solution just before use, by adding 400 mg alpha-tocopherol and 30 mg cholesterol to 1.0 ml of the extracted lipids from Exercise 6.6. 5
2. Make a chamber from a teflon cup by piercing the cup with a 1-1.5 mm hole, and placing the cup into a slightly larger polystyrene beaker.
3. Fill both the teflon cup and the outer chamber with 0.1 M NaCl.
4. Use a fine sable brush (3-5 hairs) to apply a small amount of the working solution to the hole in the teflon cup. Be careful to spread the lipid solution evenly but do not puncture the membrane which will form.
5. Observe the characteristics of the membrane formation visually.

   The solution will spread over the hole, then begin to recede and form a gradually thinning membrane. As it does so, the optical properties will change, especially if viewed with reflected light with a simple hand held magnifier (10-20X). The membrane will demonstrate interference colors as it begins to thin and will ultimately become thin enough to become completely transparent. As it becomes transparent, it becomes known as a black membrane.

6. Using an interference scale, plot the thickness of the membrane over time during its formation.

Exercise 6.7 - Electrical Properties of Artificial Membranes

LEVEL II

Materials

- Artificial lipid membrane from Exercise 6.6
- KCl electrodes 6
- DC voltage source
- Wheatstone bridge to monitor resistance 7

Procedure

1. Insert a salt bridge connected to standard electrodes and suitable for measuring both resistance and potential differences across the membrane.
2. Measure the resistance of the synthetic membrane by passing a small DC current through the membrane and measuring the drop in voltage.
   If one knows the applied voltage (100-200 mV) and the measured voltage (Δ mV), the following formula will give the resistance (Ohm's law):

$$R_{m} = (E_{m} / (E_i - E_{m})) * R_i$$

where R_i is the series resistance

E_i is the calibrated input voltage

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E_m is the voltage appearing across the membrane.

3. Calculate the area of the membrane based on the diameter of the drilled hole, and multiply R_m (measured resistance) by the area of the membrane. This will give a normalized resistance in ohms-cm^2.
4. Compare your calculated value to the data in Table 6.4.

Table 6.4 - Electrical resistance of membranes

BLM Source	Aqueous phase	Resistance (Ω/cm2)
Brain lipids + a-tocopherol	0.1 N NaCl	10^7-10^8
Egg lecithin	0.1 N NaCl	10^8
Egg lecithin	Various	10^8-10^{10}
Cephalin + cholesteryl oleate + a-tocopherol	0.1 N NaCl	10^7-10^8
Egg lecithin	Various 0.001 N NaCl 0.001 N NaI	10^7 10^6-10^8 10^4
Oxidized cholesterol	0.1 N NaCl	10^8-10^9
Glycerol distearate	0.1 N NaCl	10^8-10^9
Cholesterol + dodecyl acid phosphate	0.1 N NaCl	10^7-10^8
Cholesterol + diotadecyl phosphate	0.1 N NaCl	10^8-10^9
Cholesterol	0.001 N NaCl + 0.008% hexadecyltrimethylammonium bromide 0.1 N NaCl + 0.008% hexadecyltrimethylammonium bromide	10^7 10^3-10^4
Retinal axon lipids + a-tocopherol	0.1 N NaCl + .005 M Tris	10^7

5. Divide your calculated value by the thickness of the membrane 8 to yield the membrane resistivity or specific resistance.

Exercise 6.8 - Measurement of Transmembrane Potential

LEVEL II

Materials

- Artificial membrane and recording apparatus from Exercise 6.6
- 1.0 M NaCl
- 1.0 M KCl

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Procedure

1. Using the recording device from Exercise 6.7, measure the voltage across the membrane with 0.1 M NaCl on both sides of the membrane.
2. Add 1.0 ml of 1 M NaCl to the external chamber and mix gently. Immediately record the changes in voltage. 9
3. When the change begins to stabilize, introduce 1.0 ml of KCl into the teflon cup and continue to monitor the voltage.
4. Graph the change in voltage across the membrane with time and the addition of first NaCl and then KCl.
5. Compare your results with the action potential of a typical vertebrate neuron.

Notes

The same apparatus as that used for measurement of resistance can be used to measure a potential, provided there is a difference in the salt concentration in the teflon cup and outside of the cup. This is best accomplished by the slight addition of a salt solution to the teflon cup, although rather elaborate procedures exist for complete substitution of the solutions (extreme care must be taken not to rupture the membrane).

In vertebrate nerve, the potential that is generated is from an influx of Na^+ followed by an efflux of K^+. These fluxes are mimiced by the addition of NaCl to the outer chamber, followed by an addition of KCl to the inner chamber.

Exercise 6.9 - Chemical Composition of Myelin

LEVEL III

Materials

- Calf brain 10
- 0.25 M sucrose
- 0.88 M sucrose
- Blender or tissue homogenizer
- Refrigerated centrifuge

Procedure

1. Homogenize 100 gms of calf brain in 0.25 M sucrose to make a 10% brei.
2. Centrifuge the homogenate at 500 xg for 10 minutes at 4° C.
3. Collect the supernatant and recentrifuge the supernatant at 11,000 xg for 10 minutes. 11
4. Discard the supernatant and resuspend the pellet in 10 ml. of 0.25 M sucrose.
5. Carefully add 25 ml of 0.88 M sucrose to the bottom of the tube, so that the resuspended pellet lies on top of the sucrose.
6. Centrifuge at 11,000 xg for 10 minutes to separate the mitochondria from the myelin vessicles. The myelin will float on the surface, while the denser mitochondria should pellet at the bottom of the tube.
7. Carefully remove the upper myelin vessicle layer.

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Exercise 6.10 - Myelin Lipid Extraction

LEVEL III

Fraction A: Cholesterol

Materials

- Myelin vessicles from Exercise 6.9
- Acetone
- Homogenizer manufactured for organic solvents
- Buchner funnel and water aspirator

All organic solvents are flammable and should be handled with extreme care. No open flames will be tolerated and all heating should be done with steam baths.

Do not use a standard household blender - the acetone will dissolve the gaskets, run into the motor and explode!

Procedure

1. To the purified myelin vessicles, add 4 volumes of acetone. Blend the mixture for 1 minute at high speed.
2. Add the blended mixture to a beaker and manually continue stirring for 5 minutes.

   Do not use any electric stirring motors.

   Filter the mixture using a Buchner funnel and cold trap attached to a water aspirator for reduced pressure. Save the filtered acetone solution.

3. Wash the residue from the filter paper back into the blender. Add 100 ml. of fresh acetone and reblend for 1 minute.
4. Add the second homogenate to the beaker, stir for 5 minutes and filter.
   Combine the two filtered acetone portions.
5. The acetone extracter material is FRACTION A. It contains crude cholesterol.

   Before storing, it should be dried to remove the acetone. This is best accomplished by flash evaporation or lyophilization. Alternatively, the solution can be allowed to evaporate in a fume hood designed to vent organic solvents. There will, of course, be some decomposition with the latter technique. Evaporate the acetone, collect the dry crude cholesterol and obtain its dry weight.

6. Keep the acetone insoluble residue (on the filter paper) for

   extraction of Fractions B and C.

Fraction B: Lecithin and Cephalin

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Materials

- Residue from extraction of Fraction A
- Petroleum ether
- Buchner funnel

Procedure

1. Extract the acetone insoluble material left over from Step 6 of the extraction of Fraction A with 200 ml of ether. Gently stir for 5 minutes.
2. Collect the filtrate using a Buchner Funnel, as above.
3. Place the ether insoluble material on the filter into a flask and re-extract with an additional 200 ml of ether. Repeat the filtration and re-extraction one final time with a third 200 ml of ether.
4. Combine all three ether filtrates.
   Save the residue for Fraction C.
5. Evaporate the ether filtrates to 50 ml under reduced pressure. Pour the concentrated extract into 200 ml of acetone and stir.
6. Collect the precipitate on filter paper and discard the filtrate. The precipitate left on the filter paper FRACTION B, containing lecithin and cephalin.
7. Obtain the dry weight of Fraction B.

Fraction C: Sphingosine Phosphatides and Glycosides

Materials

- Residue from extraction of Fraction B
- Ethanol

Procedure

1. Extract the ether insoluble residue obtained in the steps above with 50 ml. of boiling ethanol. Vacuum filter the hot ethanol while it is still hot. Discard the residue.

   Use steam heat only and work in a laboratory properly equipped for organic extractions. Hot ethanol has a very low flash point - it will explode!

2. Cool the ethanol filtrate and collect the precipitate by vacuum filtration. This precipitate is Fraction C.
3. Obtain the dry weight of Fraction C.
4. Determine the percentage of each Fraction dry weight relative to the original wet weight of calf brain. Dissolve each of the Fractions to a final concentration of 1% (w/v) in chloroform:methanol (3:1) before further analysis.

Exercise 6.11 - Quantitative Analysis of Lipid Classes by TLC

LEVEL III

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Two methods of lipid analysis are commonly used. The first involves isolation of classes of lipids, followed by Thin Layer Chromatographic separation and quantitation directly on the chromatographic plates. The second method involves the isolation of the components on the TLC plate, followed by conventional quantitative methods.

The lipid classes are divided into:

1. Neutral lipids (triglycerides)
2. Polar Lipids (phosphoipids)
3. Cholesterol

Ordinarily, the neutral lipids are analyzed first, as they are readily separated with one dimensional TLC systems. The polar lipids require two dimensional TLC analysis, and cholesterol needs to be analyzed separately.

The lipids may be chromatographed and measured for the following:

1. Melting Point
2. Salkowski Test (color developed with H_2SO_4)
3. Liebermann-Burchard Test 20 (test for 3-hydroxysteroids using acetic anhydride)
4. Digitonide Derivative (precipitates cholesterol)
5. Charring (spraying with an oxidant and heating to carbonize the lipid)

A typical analysis would involve two dimensional chromatography followed by charring. Individual lipids would be identified by the Rf values of the spots on the chromatograms and could be quantitated densitometrically.

Endnotes

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1. Modified from Giese, A.C. 1964. *Laboratory Manual in Cell Physiology* Revised ed., p 67-68. Boxwood Press, Pittsburgh.
2. Collect Amoeba by centrifugation three days prior to use, wash, and resuspend without food. It is important that the Amoeba be starved for this exercise.
3. If the dye is too strong, and the Amoeba are stained intensely blue, dilute the dye 1/10 or even 1/100.
4. This material is readily available from local slaughter houses. If there is not one convenient to your location, and a local butcher will not help, rat brains can be substituted. Quantities will need to be adjusted accordingly.
5. This solution is stable for about 5 days if refrigerated, and can be prepared ahead and used for multiple lab sessions.
6. A salt bridge is simply a saturated KCl solution (in 2% (w/v) agar) placed within glass, polyethylene, or teflon capillary tubes and with platinum electrodes or commercially available calomel electrodes immersed within the agar. Thus, they bridge the gap between the solution being monitored and the rather large wire connections of a recording electrode.
7. A suitable measurement can be made by using the millivolt potential scale on a good pH meter. It may

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require some assistance from the local physical chemist, or a physicist to construct the proper electrodes.

8. Assume a thickness of 5 x 10^{-7} cm if it was not measured.
9. This will model the normal distribution of increased external Na^+ observed in vertebrate neurons. As the Na^+ diffuses into the center of the teflon cup, it will model the influx of ions associated with an action potential.
10. Yields of myelin will vary depending upon the developmental age of the animal and upon the species. Adult rat brains will yield about 40-90 mg. of pure myelin per brain while the white matter from bovine brain will yield about 100 mg of pure myelin per gram of white matter. The purity will depend upon whether whole brain is used or just the white matter, with the latter yielding better results. Whole brains would contain a fair amount of microsomal membranes isolated along with the myelin membranes, and thus would have more impurities.
11. The pellet contains mitochondria and myelin vessicles.

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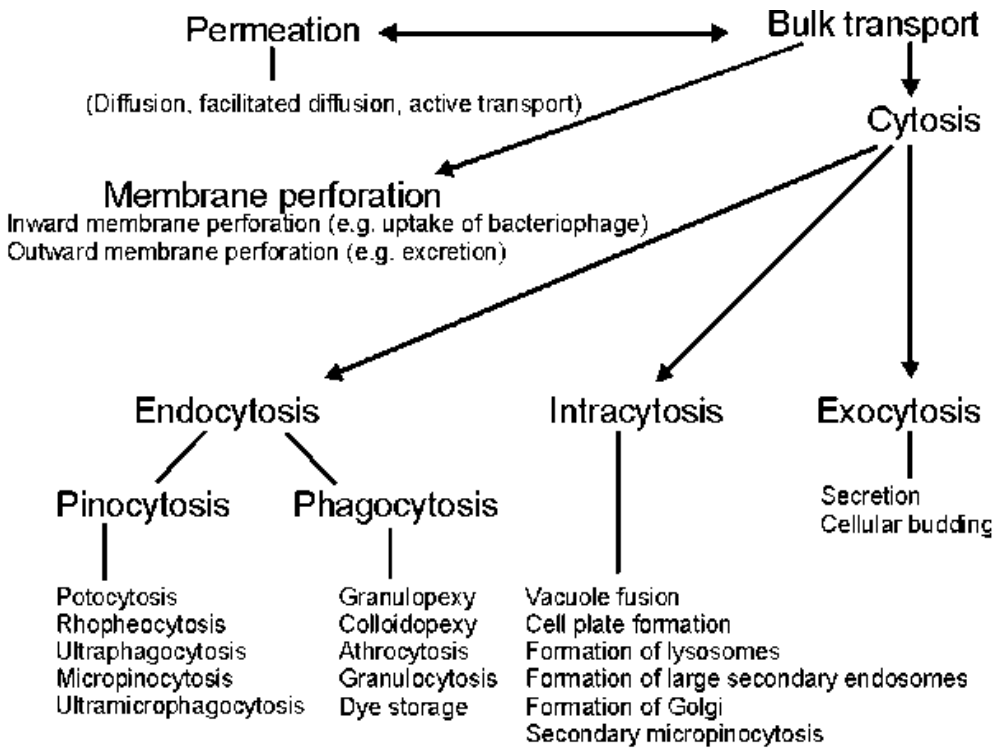
Chapter 7: Microsomes

Introduction:

Figure 7.1 Vacuome concept



The use of homogenization techniques and differential centrifugation has made possible the study of many isolated, membrane bound organelles. The nucleus, mitochondria and various plastids were obvious targets for analysis, since they are relatively large, and are visible with the light microscope.

Smaller structures, and particularly those associated with internal membranes, were more difficult to analyze. In 1931, W.H. Lewis observed the uptake of neutral red dye into cell vacuoles, 1 and coined the term PINOCYTOSIS, literally cell drinking. Lewis was interested in the small membrane bound organelles found within plants cells, and named vacuomes by the French Botanist, P.A. Dangeard in 1919. The term vacuome described a structure or structures in plant cells which were associated with the tonoplast (the membrane surrounding a large central vacuole) and which was apparently part of an internal membrane system of communication and flow. Figure 7.1 presents an extended view of the vacuome concept, which was developed to explain both pinocytosis and its related phagocytosis, cell eating . The study of vacuomes was an early attempt to define the function of internal membrane bound vessicles as components of membrane flow.

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Several years later, the concept of an organized system of internal membranes was extended to animal cells. Our perspective on membrane bound vessicles was altered significantly in 1949 when Christian DeDuve analyzed the activity of the enzyme acid phosphorylase during a study of insulin activation. 2 Acid phosphorylase is a useful indicator of enzymatic digestion in cells. DeDuve was interested in how cells digested substances which were either pinocytized or phagocytized, and specifically, what the relationship of digestion was to the small vacuoles, MICROSOMES, which could be isolated via centrifugation. As DeDuve explains, the lab purchased an expensive and new ultracentrifuge capable of isolating small particles and even molecules. DeDuve discovered that acid phosphorylase was associated with specific microsome fractions and that the activity of the enzyme could be released by the actions of blenders, hypotonic media, freezing/thawing or detergents. As work progressed, several other enzymes were localized within these microsomes. In 1955, DeDuve et al published the now classic paper describing for the first time, a new organelle, the lysosome . 3 DeDuve was later to be awarded a Nobel Prize for this pioneering work.

The lysosome is one type of microsome; its role in cell digestion brought together a story of internal membrane flow which is integrated with the twin processes of ENDOCYTOSIS and EXOCYTOSIS of the plasma membrane. The lysosome was seen as a part of an elaborate system, that could be fractionated, but which only made sense in the whole. Pinocytosis, Phagocytosis, Golgi Complex, Endoplasmic Reticulum and Plasma Membrane where all part of this system. Figure 7.2 presents our current concept of how a cell's internal membranes are organized.

The exercises in this chapter deal with the isolation and analysis of different types of microsomes. Careful homogenization of the cell is critical for the subsequent separation of microsome fractions. Lysosomes, for example, can be isolated from rat liver in either an isosmotic sucrose media (0.25 M), or in a hyperosmotic sucrose (0.88 M). With the use of isosmotic solutions, mild homogenization is required to prevent rupturing of the lysosome. Excessive membrane damage also results in the creation of high levels of contaminating vessicles formed from broken plasma membranes, endoplasmic reticulum, and nuclear envelopes.

The physical properties of isolated organelles can be determined and their function inferred from their chemical compositiion. Table 7.1 lists some of the properties of microsomes isolated in isosmotic media.

Table 7.1 Physical properties of microsomes

	Mitochondria	Lysosome	Perosisome
Identifying enzyme Relative volume (cm³/g dry matrix)	Cytochrome oxidase	Acid phosphatase	Urate oxidase
Matrix	0.760	0.769	0.756
Hydration	0.430	0.256	0.214
Sucrose space	0.905	1.075	2.510
Osmotic space	0.595	0.485	0
Total	2.690	2.585	3.480

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Relative mass (g/g dry matrix)			
Matrix	1.000	1.000	1.000
Hydration	0.430	0.256	0.214
Sucrose space	0.936	1.112	2.596
Osmotic space	0.595	0.485	0
Total	2.961	2.853	3.810
Density	1.099	1.103	1.095
S 0.25 M/S 0.70 M sucrose			
2.74	2.62	2.62	

7.1 Lysosome Isolation in Isotonic Sucrose

LEVEL I

MATERIALS

- Rat liver
- Physiological saline (0.85% w/v NaCl)
- 0.25 M sucrose in 10 mM Tris-HCL, pH 7.4
- Brendler teflon homogenizer
- Refrigerated preparative centrifuge
- 0.08 M $CaCl_2$ in 0.25 M sucrose plus 10 mM Tris-HCl
- 150 mM KCl in 10 mM Tris-HCl Buffer, pH 7.4
- Phase contrast microscope, slides, coverslips

PROCEDURE [4]

1. Decapitate and exsanguinate a rat that has been starved for at least 24 hours prior to the lab. [5]

   Fill a syringe with saline and gently perfuse the liver by forcing the saline through the hepatic portal vein, and through the liver.

2. Remove the liver, place it in a preweighed beaker and weigh the beaker and liver. Calculate the weight of the liver.
3. Prepare a 10% (w/v) homogenate or brei. For each gram of liver, add 9.0 ml of 0.25 M sucrose in 10 mM Tris-HCL, pH 7.4. to the beaker.
4. Gently chop the liver in the sucrose and transfer the chopped liver to a teflon homogenizer. [6]

   Gently homogenize the liver while keeping it chilled.

5. Centrifuge the brei at 12,000 xg for 10 minutes at 4 ° C. Decant the supernatant into a chilled beaker and discard the pellet.

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6. Add 0.08 M $CaCl_2$ to the supernatant to yield a final concentration of 8 mM (use 1 ml of $CaCl_2$ per 9 ml of supernatant). Stir gently and recentrifuge at 25,000 xg for 15 minutes at 4 ° C
7. Carefully remove and discard the supernatant. 7
8. Resuspend the pellet (containing the lysosomes) in 30 ml of 150 mM KCl in 10 mM Tris-HCl Buffer, pH 7.4.
9. Re-sediment the lysosomes by a final centrifugation at 25,000 xg for 15 minutes at 4 ° C.
10. Remove a small portion of the pellet for Exercise 7.2. Resuspend the remainder of the pellet in 30 ml of 150 mM KCl/10 mM Tris-HCl Buffer. This suspension is the lysosome fraction for Exercise 7.3.
11. Prepare a wet mount of the resuspended lysosome pellet and observe with a phase contrast microscope at 100X. Draw any structures observed.

7.2 Em observations of microsomes

LEVEL I

Figure 7.3 Isolated microsomes

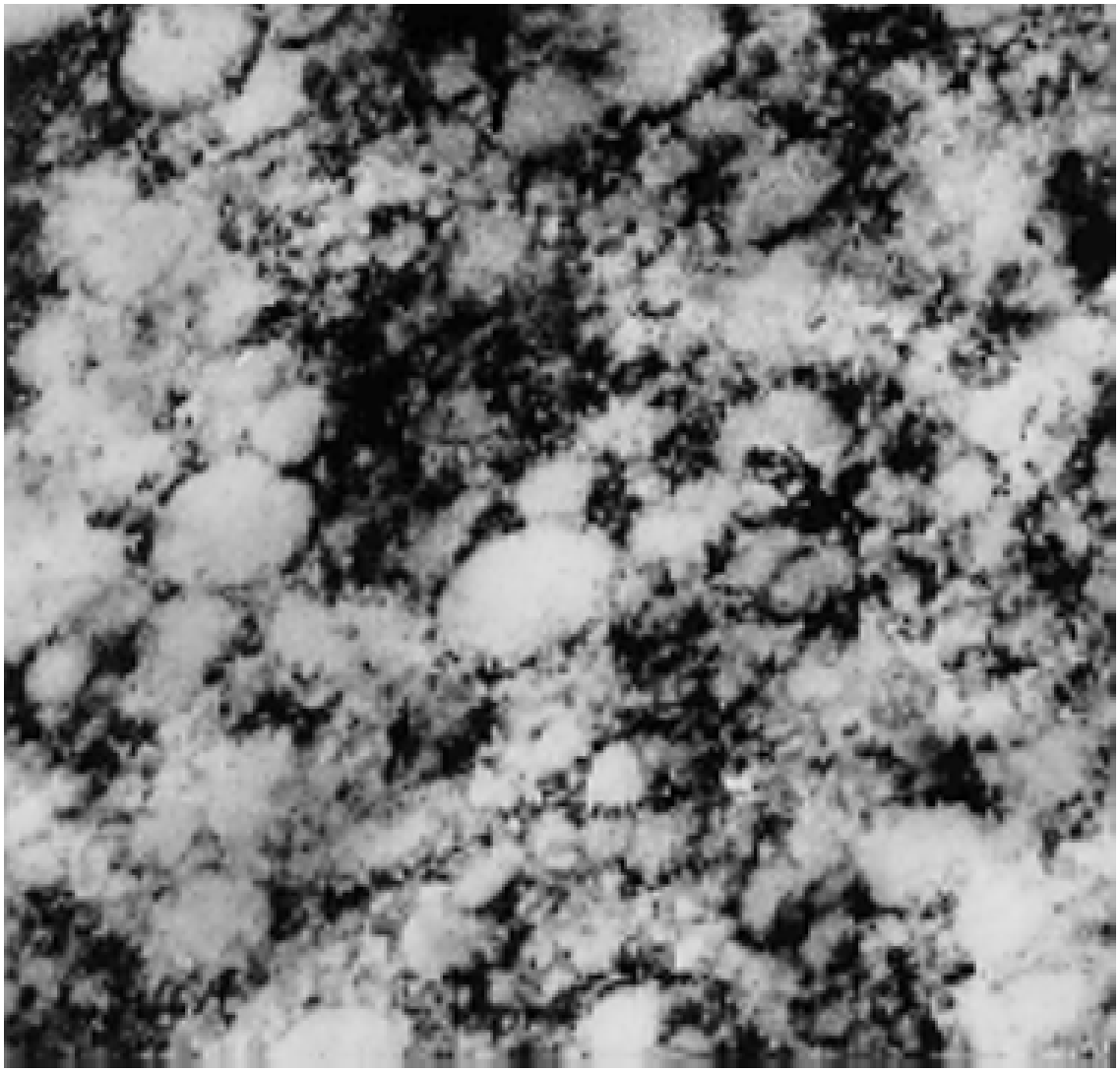

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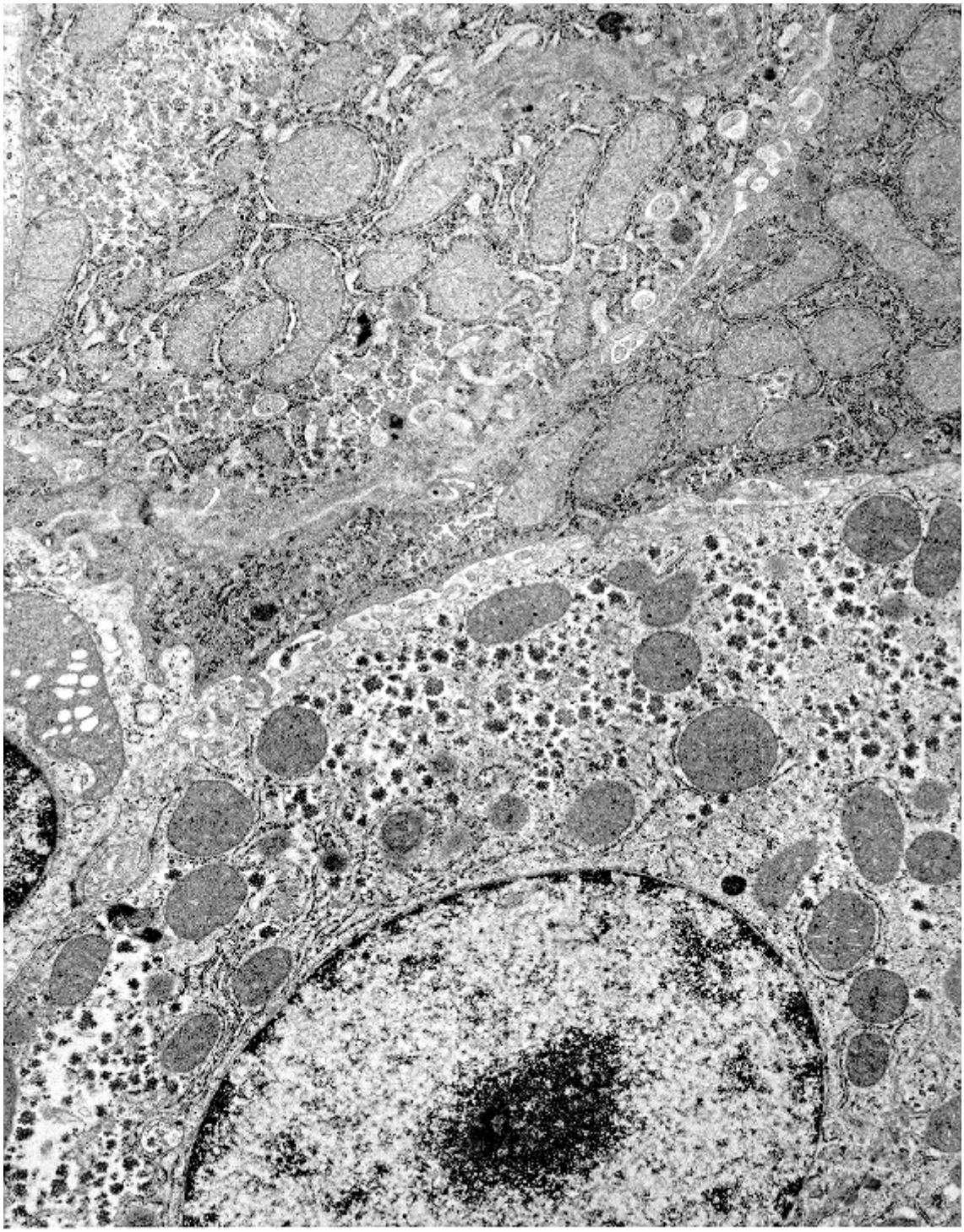
Figure 7.4 TEM of rat hepatocyte



MATERIALS

- 1% Glutaraldehye (GTA)
- 1% Osmium tetroxide
- Epoxy or vinyl resin for TEM
- TEM photomicrograph of liver cells

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- Transmission electron microscope

PROCEDURE

1. Place a mm³ piece of the final pellet from Exercise 7.1 into a small vial containing 1% GTA. Fix the pellet for 2 hours.
2. Rinse the pellet with water and place in three changes of water for 30 minutes each, to remove any residual GTA.
3. Post fix the lysosome pellet in 1% osmium tetroxide for 1 hour. Wash thoroughly with 3 changes of water (30 minutes each).
4. Dehydrate the tissue by passing through a series of graded alcohols or acetone, and embed in plastic blocks.
5. Section the plastic blocks and place on coated grid. Double stain with uranyl acetate and lead nitrate and examine with a transmission electron microscope.
6. Compare the view of the compacted lysosome pellet to the structure and distribution of microsomes in an intact hepatocyte (liver cell). Use figures 7.3 and 7.4 for reference.

7.3 Chemical chracterization - acid phosphatase

LEVEL I

Table 7.2 Chemical components of microsomes

Composition	RNA, ribosomal and others
Protein (variable), both enzymes and structural	Lipids (30-50% of composition weight) Phospholipids Phosphatidylserine Cholesterol Lecithins Phosphatidyl inositol Unsaturated Fatty acids
Protein metabolism	Several enzymes for protein synthesis Iodine incorporation enzymes Peptidases, amidases
Fatty acid metabolism	Reductive synthesis of short chain fatty acids Acylation with Acyl-CoA (ligase) Synthesis of glycolipids and plasmalogens Neutral glyceride synthesis
Phospholipid metabolism	Phosphatidic acid synthesis Decarboxylation of phosphatidylserine Redistribution of phosphatides in membranes Phosphatidylcholine synthesis Transmethylation of phosphatides

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Steroid metabolism	Synthesis of cholesterol from acetate Interconversion and degradation of steroids Hydroxylations, aromatizations, dehydrogenases and hydroxylases
Nucleotide metabolism	Nucleoside diphosphatases NAD and NADH pyrophosphatase, NADase 5'-Nucleotidases
Oxidation-Reduction	NADH and NADPH cytochrome C reductase Nonspecific and NADP diaphorase Pyridine nucleotides Cytochromes, P450, coenzyme Q
Transport and Transmission	Sarcoplasmic AMPases Cholinesterase Mg++ Ca++ dependent AMPases
Drug detoxication	N- and O- deacylations N-demethylation, sidechain and thio-ester oxidation Desulphuration, deamination and deiodination Hydroxylation of azo dyes
Binding properties	Azo dyes Iodide Epinephrine, norepinephrine

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MATERIALS

- 1 M Sodium acetate buffer, pH 5.7
- 0.1 M $MgCl_2$
- 0.05 M p-Nitrophenyl phosphate
- Microsome fraction from Exercise 7.1
- 150 mM KCl in 10 mM Tris-HCl Buffer, pH 7.4
- 0.5 N KOH
- Water bath at 30 ° C
- Spectrophotometer and tubes
- Bradford Protein assay

PROCEDURE

1. Prepare a series of six test tubes containing 0.5 ml of each of the following (total of 1.5 ml):

   1 M Sodium acetate buffer
   0.1 M $MgCl_2$
   0.05 M p-Nitrophenyl phosphate

2. Add 3.3 ml of distilled H_2O to each of the six tubes. Mix well, and place in a 30 ° C water bath to temperature equilibrate.
3. Prepare a serial dilution of your lysosome fraction by adding 1.0 ml of lysosome suspension from Exercise 7.1 to 9.0 ml of 150 mM KCl/10 mM Tris-HCl Buffer. Mix well and add 1.0 ml of the diluted

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suspension to a new tube containing 9.0 ml of Tris- HCl buffer. Mix and repeat the dilutions two more times. In addition to the undiluted lysosome suspension, label the diluted fractions as 1/10, 1/100, 1/1,000 and 1/10,000.

4. Add 0.2 ml of Tris-HCl buffer and 2.0 ml of KOH to tube #1 from step 1.
5. Turn on a spectrophotometer, adjust the wavelength to 405 nm and use the sample in tube #1 to blank the instrument.

6. Add 0.2 ml of the undiluted the lysosome fraction to tube #2, mix and place in water bath at 30 ° C for exactly 5 minutes.

6. Add 2.0 ml of KOH to tube #2, mix and immediately read the absorbance of the solution at 405 nm.
7. The absorbance should be between 0.3 and 0.4. If not, repeat steps 6 and 7 for the diluted samples, starting with the 1/10 in tube #3 and continuing through the 1/10,000 dilution in tube #6. Each should be done separately, one at a time to prevent incorrect timing of the reaction.
8. Using the absorbance reading for the dilution which yields an absorbance change of 0.3-0.4 in five minutes, determine the actual rate of absorbance change for that dilution.

10. Convert the absorbance change to a rate of p-Nitrophenyl phosphate conversion to p-nitrophenol. Use the Beer-Lambert law, with an extinction coefficient = 18.8 x 10⁶ Abs. Units/mole of p-nitrophenol.

Convert all absorbance readings to micromoles of p-nitrophenol formed in five minutes. Divide by the time (5 minutes) to calculate micromoles formed per minute.

9. Determine the concentration of protein in the dilution from step 9, using the Bradford protein determination (Appendix G) and bovine serum albumin as the standard.
10. Determine the rate of enzyme activity per mg. protein present in the diluted fraction.

Tube/Dilution	Protein Content	Absorbance(5')	[p-nitrophenol]	Activity/minute
1. Blank	____	0	____	____
2. 10⁰				
3. 10⁻¹				
4. 10⁻²				
5. 10⁻³				
6. 10⁻⁴				

OPTIONAL

Enzyme activity and protein concentration can be measured for each step in the centrifugation and isolation of lysosomes. Assuming that acid phosphatase is an effective measure of the purity of lysosomes, the increased purity during separation can be monitored by measuring the increased enzyme activity per mg of protein. Lysosome purification can be monitored to yield the maximum activity per mg. protein. While lysosomes are best characterized by the presence of acid pyrophosphorylases, they contain a large number of functional enzymes.

Table 7.2 presents a list of chemical constituents found in isolated lysosomes.

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7.4 Chromaffin granules from adrenal glands

LEVEL II

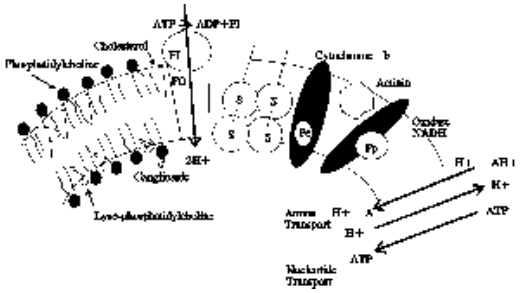

Figure 7.5 Schematic membrane of chromaffin granule

Table 7.3 Composition of chromaffin granules

Protein	NADH oxideoreducatase F1-H+-translocating ATPase Phosphatidylinositol kinase Synaptin Enkephalin Dopamine-B-hydroxylase Cytochrome b Glycoprotein Chromogranin
Lipid	Phospholipid Cholesterol
Catecholamine	Dopamine Epinephrine Norepinephrine
Nucleotides	ATP, GTP, UTP, ADP, AMP
Metal ions	H+, Ca++, Mg++, Na+, K+, Cu++, Fe++
Mucopolysaccharides	
Gangliosides	
Ascorbic acid	

MATERIALS

- Bovine adrenal glands (preferred fresh from slaughterhouse)
- 0.27 M Sucrose, 10 mM Tris-maleate buffer, pH 7.0
- 30% Percoll (v/v) in 0.27 M sucrose, 10 mM Tris-maleate, pH 7.0
- Refrigerated, preparative centrifuge with swinging bucket rotor for 50 ml tubes
- Fraction collecting device
- ATP-luciferase kit [8]

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- UV/VIS spectrophotometer
- Optional
    - Lowry protein assay
    - Catecholamine assay
    - TEM fixatives and embedding materials

PROCEDURES [9]

1. Dissect out the medulla of an adrenal gland, weigh and quickly mince.
2. For each gram of tissue, add 5 volumes of chilled 0.27 M sucrose, 10 mM Tris-maleate buffer, pH 7.0 and homogenize in a Potter-Elvijem homogenizer.
3. Centrifuge the brei for 5 minutes @ 1,250 xg @ 4 ° C . Discard the pellet and recentrifuge the supernatant @8,650 xg @4 ° C for 35 minutes.
4. Collect the second pellet (pink, granular) and wash by resuspending in fresh sucrose and recentrifuging @8,650 xg as above.
5. Resuspend the washed pellet to yield a final protein concentration of 60-80 mg/ml (as measured by OD_{280}).
6. Add 38 ml of 30% Percoll-sucrose in tris-maleate buffer to a 50 ml clear polycarbonate centrifuge tube and centrifuge the Percoll for 5 minutes @20,200 xg to form a gradient.
7. Add 2.0 ml of the resuspended chromaffin granule pellet to the top of the preformed Percoll gradient and centrifuge for 40 minutes @ 8,700 xg @ 4 ° C.
8. Fractionate the centrifuged chromaffin granules into 1.0 ml fractions. Measure the absorbance of each fraction at 650 nm.
9. Measure the ATP content of each fraction using a commercially available luciferase reaction.
10. Plot the fraction number vs the concentration of ATP.

OPTIONAL

Measure catecholamines by spectrophotometric analysis of adrenochrome formation at 480 nm, using norepinephrine as a standard. [10] Repellet the chromaffin granules and prepare a sample for electron microscopic observation. Monitor mitochondrial and lysosomal contamination via the presence of cytochrome oxidase and acid phosphatase, respectively. Determine total protein content by the Lowry method.

NOTES

Adrenal medullary tissue consists of cells containing chromaffin granules (named for their ability to stain with chromium stains) which are 100-300 nm in diameter. These granules consist of the catecholamine hormones epinephrine and norepinephrine (20% by weight), ATP (15%), protein (35%) and lipid (20%). About 85% of the granules store epinephrine, while the remainder store norepinephrine. Table 7.3 presents a more detailed chemical composition of chromaffin granules.

The adrenal medulla from cows and pigs has long served as a source of material for the isolation of specialized organelles which were originally believed to be storage vessicles for the catecholamines. These organelles are known as chromaffin granules and are embryologically related to the adrenergic neurotransmittor granules of the central nervous system. Lately, the presence of a proton pump has been identified within these granules and extensive studies are underway to relate the

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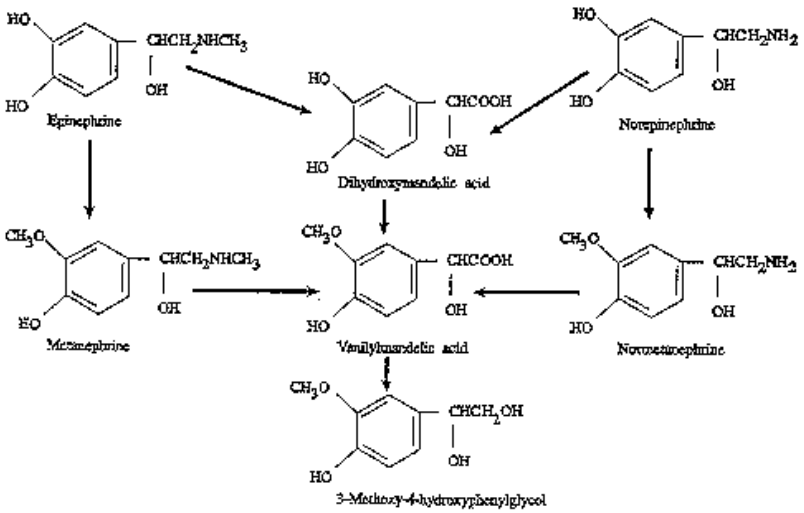
chemiosmotic concepts of membrane transport to the synthesis and release of the biologically active catecholamines. 11

A schematic of a chromaffin granule membrane is presented in figure 7.5.

7.5 Measurement of catecholamines

LEVEL III

Figure 7.6 Catecholamine metabolism



Original source: R.M. Berne and M.N. Levy. **Physiology**. C.V. Mosby Co. 1983, p. 1062.

The function of chromaffin granules is the synthesis of the catecholamines. Thus, we can analyze chromaffin granule function by the level of catecholamines found in isolated granules; in particular dopamine, epinephrine and/or norepinephrine can be monitored. Unfortunately, these compounds are somewhat difficult to measure. While there are several means of monitoring these compounds, 12 the primary procedure requires the use of radio-immunoassay. 13

As a reference, figure 7.6 illustrates the interaction of the catecholamine hormones..

Endnotes

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1. Lewis, W.H. 1931. BULL. JOHNS HOPKINS HOSP. 49,17.
2. According to the words of C. DeDuve in The Lysosome in Retrospect in LYSOSOMES IN BIOLOGY AND PATHOLOGY Vol. 1 (J.T. Dingle and H.B. Fell, eds.) North-Holland Publishing, Amsterdam, 1969.

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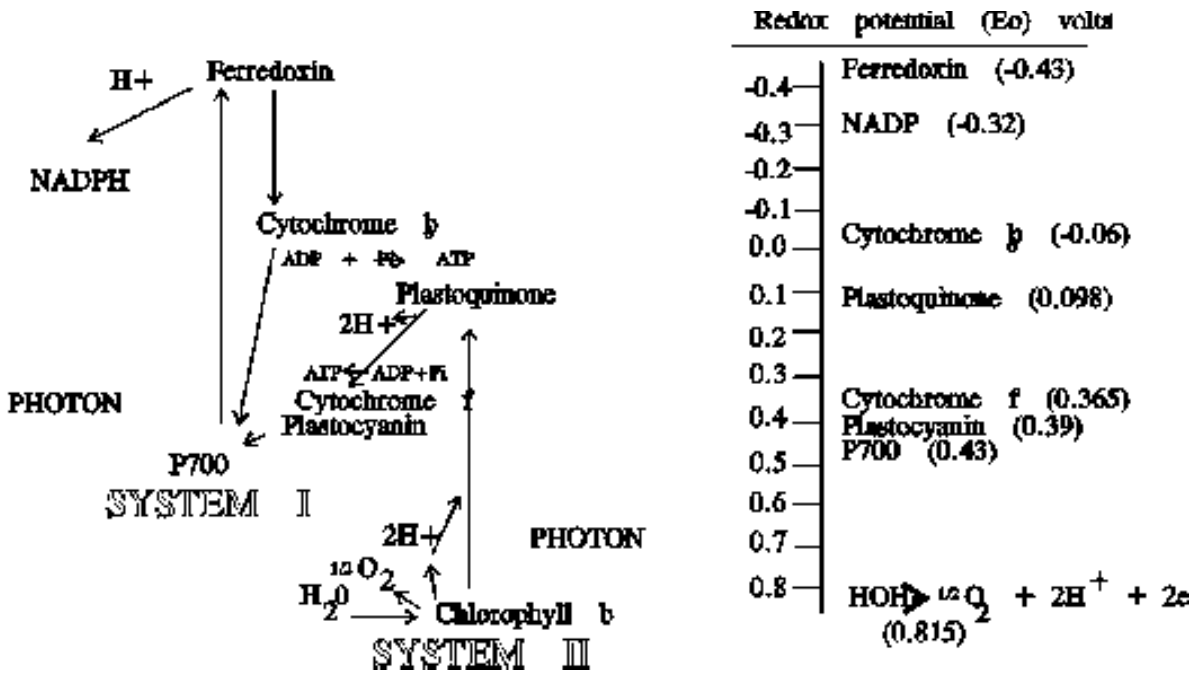
Chapter 8: Photosynthesis\Respiration

Introduction

The physiological reactions of mitochondria and chloroplasts can be reduced to a series of electron transfers, catalyzed by specific enzymes found within the organelles. Thus, we can study the component processes of photosynthesis and respiration by isolating the organelles and measuring specific enzyme activity associated with that organelle.

Photosynthesis requires two processes which can be functionally dissociated, although they work as a unit within the chloroplast. The first process is known as the light reactions, while the second is known by analogy as the dark reactions. The light reactions are rapid changes in the subatomic arrangements of molecules that ultimately split water in the presence of light (photodissociation). Hydrogen atoms from the water are used to reduce NADP to $NADPH_2$, and are then used to reduce CO_2 to CH_2O.

Figure 8.1 Light reactions of photosynthesis



The photodissociation of water in the presence of chloroplast fragments is known as the Hill Reaction. It results from the physical capture of light quanta (energy) into the electron orbit of chlorophyll molecules and the subsequent transfer of an "excited" electron to the orbit of an adjacent molecule. The end products of this reaction are free hydrogen, oxygen and electrons. The electrons

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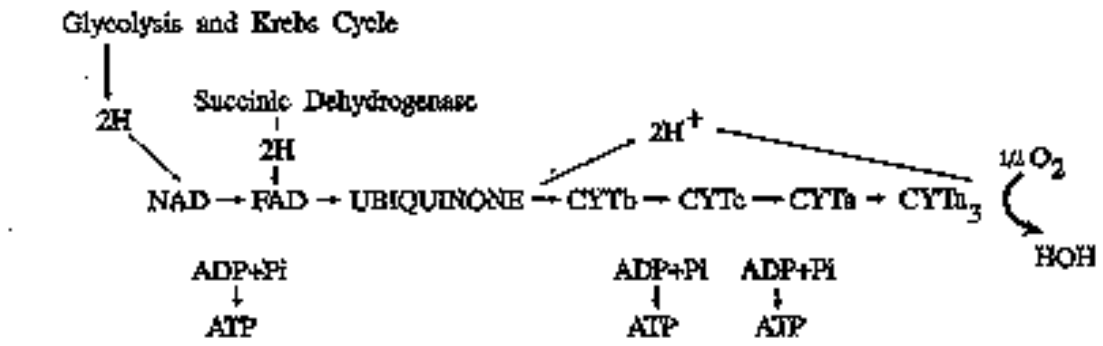
are utilized for further chemical reduction reactions, the hydrogen becomes the ultimate hydrogen acceptor in the reactions; and oxygen is a by-product.

Figure 8.1 presents the light reactions, along with the redox potentials of the compounds involved. Complete details of the cyclic and noncyclic pathways of the light reactions are beyond the scope of this manual.

For our purposes, however, the Hill reaction can be monitored by the addition of an electron accepter to the system, and one which will more readily accept the electron. If the electron acceptor is a pigment which alters its color when reduced (by gaining an electron), a simple colorimetric analysis can be used to monitor the photodissociation of water. Basically, the reducing power generated by splitting the water can be used to reduce a dye such as 2,6-dichloroindophenol, which is blue when oxidized, and colorless when reduced.

In respiration, a similar type of oxidation/reduction occurs, except that the process is not physical, but chemical (i.e. it is temperature dependent and slower than a physical reaction). Molecules such as succinic acid are oxidized within the Kreb's Cycle by the removal of both a hydrogen atom and a corresponding electron. Succinic acid dehydrogenation produces fumaric acid; the enzyme performing this reaction is succinic dehydrogenase, and the hydrogen with its electron is normally transferred to NAD.

Figure 8.2 Electron transfer system



These reactions are associated with the inner membranes of the mitochondria, and specifically to structures known as respiratory particles. Within these fragments of the inner membrane, the electron transport system functions and the hydrogen and its electron are dissociated. The electron passes through a series of respiratory pigments (the cytochromes) and combines with molecular oxygen through the use of the enzyme cytochrome oxidase. The electron transfer system is outlined in Figure 8.2.

The activities of two enzymes, succinic dehydrogenase and cytochrome oxidase, can be monitored in a manner similar to that for the Hill reaction of photosynthesis. A dye intermediate can be introduced to intercept the electron from the ETS. The gain of electrons will reduce the dye which will consequently change color. The color change may be monitored spectrophotometrically.

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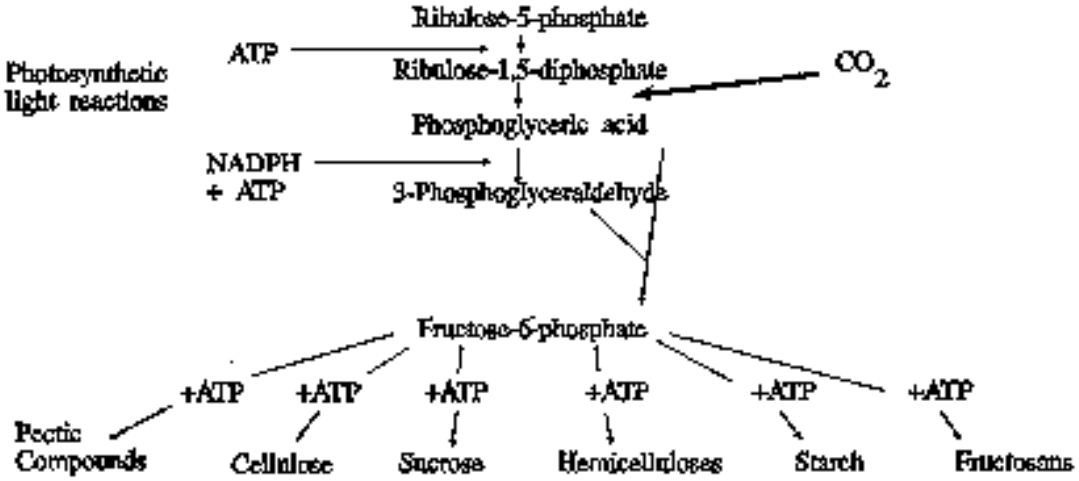
Figure 8.3 C3 metabolism (Calvin Benson Cycle)



The dark reactions of photosynthesis behave differently than the light. They are chemical reactions catalyzed by enzymes and are thus slower than the physical reactions of the light reactions. As chemical reactions, they are temperature dependent. The C3 and C4 pathways of the dark reactions are given in Figures Figures 8.3 and 8.4, respectively.

The chemical reactions of respiration are divided into two major pathways, glycolysis and the Kreb's cycle. These pathways are presented in Figure 8.5. Examination of Figures 8.3 through 8.5 will demonstrate many similarities in the biochemistry of energy metabolism, whether within chloroplasts or mitochondria. Note that glycolysis occurs within the cytoplasm of cells whereas Krebs reactions occur within the mitochondrial stroma. C3 metabolism is within a single plast cell with reactions occuring in the cytoplasm as well as within the chloroplast. C4 metabolism is a process that requires several cells, with distinct compartmentalization of function on a tissue level.

Mitochondria and Chloroplasts can be extracted by gentle rupture of the pertinent cells and differential centrifugation in a media designed to maintain the osmotic and functional integrity of the organelles. The activity of the organelles will vary significantly with the source, the age, and such factors as the length of time of storage before extraction. The organelles can be kept for several days in cold storage, but will gradually degenerate and decompose. It is easier to work with isolated intact organelles than directly with quantosomes or oxysomes, which are unstable under laboratory conditions.

Figure 8.4 C4 metabolism

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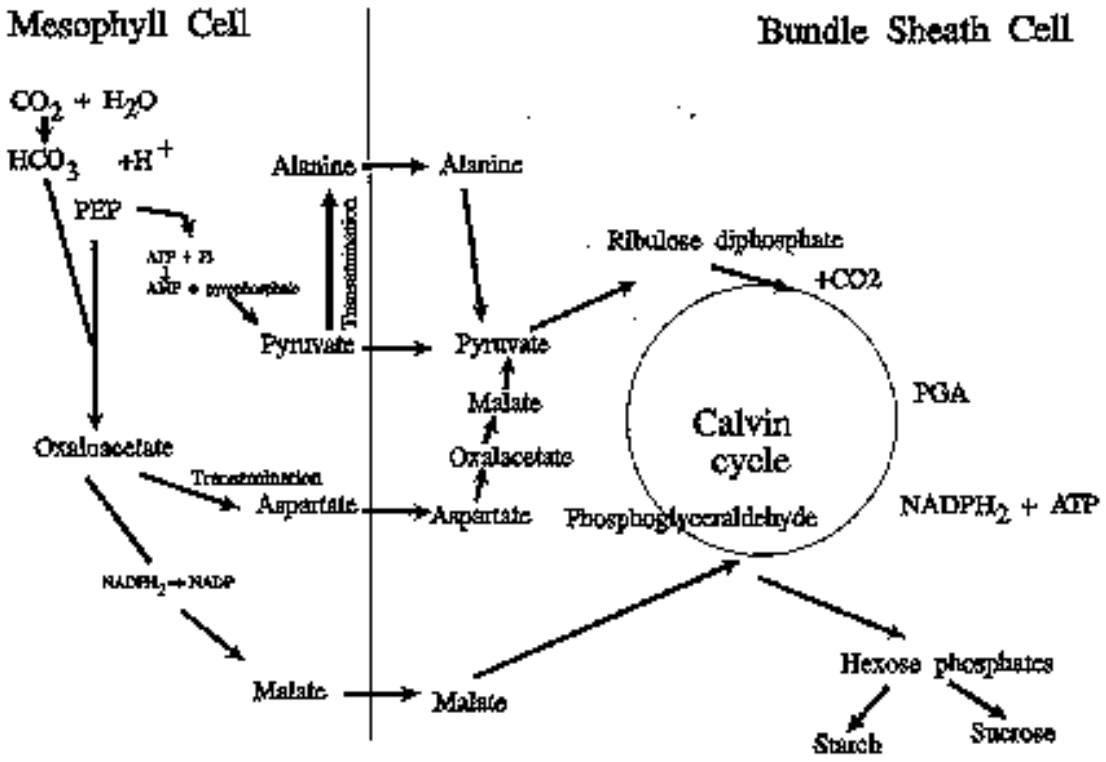

Figure 8.5 Glycolysis and the Krebs cycle

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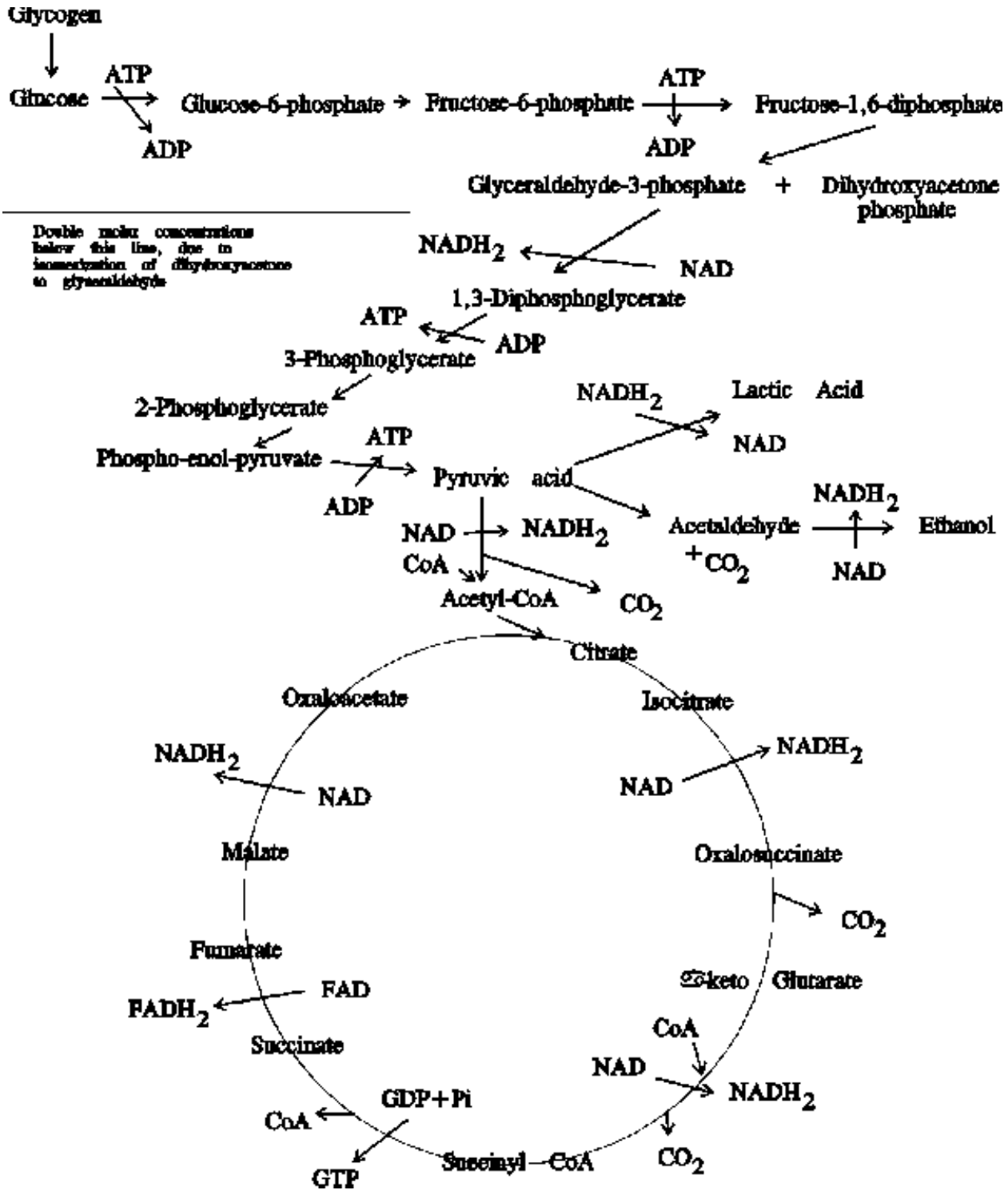

Exercise 8.1 - Isolation of Chloroplasts from Spinach Leaves

LEVEL I

Materials

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- Fresh spinach leaves
- Grinding solution
  - 0.33 M Sorbitol
  - 10 mM Sodium pyrophosphate ($Na_4P_2O_7$)
  - 4 mM $MgCl_2$
  - 2 mM Ascorbic Acid
  - Adjust pH to 6.5 with HCl
- Chopping board and knife
- Chilled mortar and pestle
- Cheesecloth
- Refrigerated preparative centrifuge
- Suspension solution
  - 0.33 M Sorbitol
  - 2 mM EDTA
  - 1 mM $MgCl_2$
  - 50 mM HEPES
  - Adjust pH to 7.6 with NaOH
- Hemacytometer and microscope

Procedure

1. Prepare an ice bath and pre-cool all glassware to be used.
2. Select several fresh spinach leaves and remove the large veins by tearing them loose from the leaves. Weigh out 4.0 grams of deveined leaf tissue.
3. Chop the tissue as fine as possible. Add the tissue to an ice-cold mortar containing 15 ml of grinding solution and grind to a fine paste.
4. Filter the solution through double layered cheesecloth into a beaker and squeeze the tissue pulp to recover all of the suspension.
5. Transfer the green suspension to a cold 50 ml. centrifuge tube and centrifuge at 200 xg for 1 minute at 4° C to pellet the unbroken cells and fragments.
6. Decant the supernatant into a clean centrifuge tube and recentrifuge at 1000 xg for 7 minutes. The pellet formed during this centrifugation contains chloroplasts. Decant and discard the supernatant.
7. Resuspend the chloroplast pellet in 5.0 ml. of cold suspension solution or 0.035 M NaCl. Use a glass stirring rod to gently disrupt the packed pellet. This is the chloroplast suspension for use in subsequent procedures.
8. Enclose the tube in aluminum foil and place it in an ice bucket. [1]
9. Determine the number of chloroplasts/ml using a hemocytometer.

Record the # of chloroplasts/ml_____

Notes

The isolation procedure used here leaves the chloroplast outer membrane intact.If you wish to study the enzymes for photophosphorylation, wash the chloroplasts and rupture the outer membranes. To rupture the outer membranes, resuspend the chloroplasts in diluted suspension solution (1:25). Immediately centrifuge the chloroplast suspension at 8,000 xg for 5 minutes to collect the

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chloroplasts. Remove the diluted suspension media and resuspend the chloroplasts in isotonic media (0.35 M NaCl or undiluted suspension buffer).

Exercise 8.2 - Chlorophyll Content

LEVEL I

Materials

- Chloroplast suspension from Exercise 8.1
- Acetone
- Spectrophotometer and tubes
- Clinical centrifuge and tubes

Procedure

1. Pipet 1.0 ml of a chloroplast suspension into a 15 ml centrifuge tube and add 8.0 ml of acetone. Mix thoroughly.
2. Add 1.0 ml of distilled water, mix again and centrifuge at 1000 xg for 3-5 minutes.
3. Turn on a spectrophotometer, adjust the wavelength to 652 nm and blank the instrument with water.
4. Measure the absorbance of the supernatant from step 2.
5. Calculate the amount of total chlorophyll in 1.0 ml of the chloroplast suspension. 2 The amount of chlorophyll is givenby the equation:

   mg chlorophyll/ml = Absorbance/34.5

6. Calculate the dilution factor for your original chloroplast suspension so that when diluted with suspension solution, the final concentration of chlorophyll will be 0.01 mg/ml.

   All subsequent work with chloroplasts will use the diluted suspension so that the criterion of step 6 is met.

7. Use a hemacytometer to determine the number of chloroplasts per ml of any diluted suspensions.

Absorbance of acetone extracted chlorophyll solution = _____
Calculated concentration of chlorophyll = _____mg chlor./ml
Dilution factor for suspension to yield 0.01 mg chlorophyll/ml = _____
Chloroplasts/ml of suspension so diluted = _____

Exercise 8.3 - The Hill Reaction

LEVEL I

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Materials

- Chloroplast suspension (Exercise 8.2)
- 0.035 M NaCl
- Boiling water bath
- Ice bath
- 5 x 10^{-7} M 3-(3,4-Dichlorophenyl)-1,1,-Dimethylurea (DCMU) 3
- 0.1 M K_2HPO_4/KH_2PO_4 Buffer, pH 6.5
- 1 x 10^{-4} M Dichlorophenolindophenol (DCPIP) 4

Procedure

1. Dilute the chloroplast suspension with 0.035 M NaCl to yield a final chlorophyll concentration of 0.01 mg/ml (Refer to Exercise 8.2).
2. Heat a 5.0 ml portion of the diluted suspension into a boiling water bath. Remove the inactivated chloroplasts from the boiling water after five minutes and place in an ice bath to cool.

   Keep the remainder of the active chloroplast suspension at 4° C (ice bath).

3. Prepare five test tubes as follows:

	1	2	3	4	5
Buffer	3.0 ml	3.0 ml	3.0 ml	3.0 ml	3.0 ml
Heated chloroplasts	1.0 ml	1.0 ml	None	None	None
Active chloroplasts	None	None	1.0 ml	1.0 ml	1.0 ml
DCMU	None	None	None	None	1.0 ml
Water	2.0 ml	1.0 ml	1.0 ml	1.0 ml	None

4. Turn on the spectrophotometer and adjust the wavelength to 600 nm. Use the solution in tube #1 to blank the spectrophotometer.
5. Add 1.0 ml of DCPIP dye to tube #2, mix by swirling and immediately measure the absorbance at 600 nm.
6. Add 1.0 ml of DCPIP dye to tubes #3, #4 and #5. Mix and immediately measure the absorbance of each. Note the time for each reading.
7. Wrap tube #4 in dark paper, and aluminum foil. This tube serves as a "dark" control and the absorbance of

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this solution will be read only at the end of the experiment. It is important that no light reach the chloroplasts inside.

8. Place tubes #2, #3, and #5 in a beaker of ice water and illuminate with a strong light source. 5
9. Measure the absorbance of each tube after 10 minutes, 20 minutes and finally after 30 minutes. Be sure to wipe the tubes dry before inserting the tube into the spectrophotometer. Record your results.

Time (Minutes)	2	3	4	5
0				
10				
20				
30				

10. Calculate the total dye reduced at 10 minute intervals using the equation:

$$Cr = Co(Ao-Ac)/ Ao$$

11. where:

    Cr is the dye reduced in moles/liter
    Co is the original dye concentration in moles/liter
    Ao is the absorbance of the mixture at the start
    Ac is the absorbance of the mixture at the end of the experiment.

12. Calculate the percent dye reduced and plot percent dye reduced per minute for tubes 2 through 5.

Exercise 8.4 - Isolation of Mitochondria

LEVEL I

Materials

- Rat, mouse or suitable source of fresh liver 6
- 0.25 M sucrose in 10 mM HEPES buffer, pH 7.5 (Homogenization buffer)
- 0.25 M sucrose in 10 mM HEPES, pH 7.5 + 1 mM EDTA (Suspension buffer)
- Teflon homogenizer
- Refrigerated centrifuge

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- Janus Green B
- Hemacytometer and microscope

Procedure

1. Sacrifice and exsanquinate a rat that has not been fed for at least 24 hours prior to lab.
2. Remove the liver and weigh it.
3. Add the liver to a beaker, and for each gram of liver, add 9.0 ml of 0.25 M sucrose in 10 mM HEPES buffer, pH 7.5. This will produce a 10% brei, a term used to indicate a homogenized suspension.
4. Add the brei to centrifuge tubes and centrifuge at 4,500 xg for 10 minutes at 4° C.
5. Decant the supernatant into clean centrifuge tubes and discard the pellet.
6. Recentrifuge the supernatant at 16,000 xg for 25 minutes at 4° C.
7. Decant and discard the supernatant. Resuspend the pelleted mitochondria in 20 ml of 0.25 M sucrose in 10 mM HEPES. Skip to step 10.

   OR

   Optional: if cleaner mitochondria are desired, resuspend in 20 ml of 0.25 M sucrose in 10 mM HEPES + 1 mM EDTA and perform steps 8 and 9.

8. Recentrifuge the suspended pellet at 16,000 xg for 25 minutes at 4° C.
9. Decant and discard the supernatant. Resuspend the washed pellet in 20 ml of fresh sucrose without EDTA and place the suspension in an ice bath until further use is required. The suspension will remain active for approximately 4-6 hours if kept cold.
10. Mix a few drops of Janus Green B solution with 0.1 ml of mitochondrial suspension. Place one drop of this mixture in a hemocytometer and determine the number of mitochondria per ml. If there are too many mitochondria to count, make serial dilutions of 1/10 to 1/1000 and recount. Diluted mitochondria must be counted rapidly. They are not stable and will decompose if not counted within a few minutes of the dilution.

    Record the number of mitochondria/ml_____

Exercise 8.5 - Respiration: Succinic Dehydrogenase

LEVEL II

Materials

- Mitochondrial suspension from Exercise 8.4
- Sodium dithionite
- 0.2 M Sorenson Phosphate Buffer, pH 7.5
- 1% (w/v) Bovine Serum Albumin 7
- 0.005 M Potassium Cyanide
- 0.00025 M Dichlorophenolindophenol
- 0.6 M Sodium succinate, pH 7.5
- 0.6 M Sodium malonate, pH 7.5

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- 0.033% (v/v) Phenazine Methosulfate in Phosphate Buffer 8
- Spectrophotometer and cuvettes

Procedure

1. Place 1.0 ml of mitochondrial suspension in a test tube and place in a boiling water bath for 5 minutes. Cool before using. This boiled preparation will allow measurement of background absorption due to the turbidity of the mitochondria when used in a spectrophotometer.
2. Prepare a series of tubes as follows:

	1	2	3	4
Phosphate Buffer	2.0 ml	2.0 ml	2.0 ml	1.0 ml
BSA	0.1 ml	0.1 ml	0.1 ml	0.1 ml
KCN	1.0 ml	1.0 ml	1.0 ml	1.0 ml
DCPIP	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Succinate	None	1.0 ml	None	1.0 ml
Malonate	None	None	1.0 ml	1.0 ml

3. Mix 2 ml of 0.00025 M DCPIP with an amount of sodium dithionite sufficient to fully reduce it. The amount of sodium dithionite is not crucial. Add a small "pinch," shake to dissolve and continue until the color of the DCPIP becomes clear.
4. Use the reduced DCPIP from step 3 to blank a spectrophotometer at 600 nm.
5. Add 1.0 ml of the boiled mitochondrial suspension and 1.0 ml of PMS to tube #1, mix by inversion and immediately place in the previously blanked spectrophotometer. Record the absorbance and continue to record at 30 second intervals for 3 minutes.
6. Add 1.0 ml of the mitochondria suspension to tube #2 and measure the absorbance immediately. Read and record the absorbance every 30 seconds for 6-7 minutes or until no further changes occur.
7. Repeat Step 6 for tube #3.
8. Repeat Step 6 for tube #4.
9. Use a hemacytometer to count the number of mitochondria per ml of suspension used.
10. Calculate the reaction rate for each tube as millimoles of dye reduced/minute/100 mitochondria.
    Convert the absorbance readings to millimoles of dye by using E $_c$= 19.1 millimoles for DCPIP @600 nm. Refer to Appendix G for details of the Beer-Lambert law.
11. Record the amount of dye reduced at 30 second intervals. Use linear regression to plot the dye reduction over time for each tube.

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Time (Minutes)	1	2	3	4
0				
0.5				
1.0				
1.5				
2.0				
2.5				
3.0				
3.5				
4.0				
4.5				
5.0				
5.5				
6.0				
6.5				
7.0				

Exercise 8.6 - Cytochrome Oxidase Manometric Analysis

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LEVEL II

Materials

- 0.01 M Potassium Phosphate Buffer, pH 7.4
- Sat. K$_3$ Fe(CN)$_6$
- 0.6 M Sodium Malonate (Malonic acid, sodium salt)
- 5 mM KCN
- 0.02% (w/v) p-phenylenediamine oxalate (PPDO) 9
- Mitochondria suspension from Exercise 8.4
- Spectrophotometer and cuvettes

Procedure

1. Prepare a series of 5 tubes as follows:

Substance	1	2	3	4	5
Buffer	3.0 ml	3.0 ml	2.0 ml	2.0 ml	3.0 ml
K$_3$ Fe(CN)$_6$	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml
Malonic Acid	None	None	None	1.0 ml	None
KCN	None	None	1.0 ml	None	None
Water	1.0 ml	None	None	None	None

2. Turn on a spectrophotometer and adjust the wavelength to 630 nm. Use tube #1 to blank the spectrophotometer.
3. Add 1.0 ml of the mitochondria suspension to tube #2 and mix by gentle inversion. Add 100 µ l of PPDO to the tube and immediately measure its absorbance at 630 nm.
4. Continue to measure the absorbance every 30 seconds for 4-5 minutes or until there is no further change in absorbance.

   Note that oxidation of p-phenylenediamine oxalate (PPDO) changes the dye from its colorless form (reduced) to purple (oxidized). Consequently, there should be a gradual increase in absorbance as the dye is oxidized, corresponding to the activity of cytochrome oxidase.

5. Add 1.0 ml of the mitochondria suspension and 100 µl of PPDO to tube #3. Measure the absorbance immediately.

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6. Continue to measure the absorbance every 30 seconds for 4-5 minutes or until there is no further change in absorbance.
7. Repeat Steps 5 and 6 with tube #4.
8. Place 1.0 ml of mitochondria suspension into a tube, and place in boiling water for 5 minutes. Cool and add to tube #5. Add 100 µl of PPDO to tube #5, invert and measure the absorbance as with tubes #2-4.
9. Record the changes in absorbance for tubes 2-5 at 30 second intervals. Plot the changes in absorbance for each tube.

Time (Minutes)	2	3	4	5
0				
0.5				
1.0				
1.5				
2.0				
2.5				
3.0				
3.5				
4.0				
4.5				
5.0				

Manometric Analysis

Each of the preceding exercises utilized the reducing power produced by the reactions of photosynthesis or respiration to monitor the color change in a dye. Another approach to the process would be to monitor the production or utilization of oxygen in the process. Since oxygen is a gas, it can be monitored through direct measurements of change in volumes of a gaseous phase. This is

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known as respirometry, and can be monitored simply (with testtubes and capillary pipettes) or by more complex and more accurate means (with the use of a Gilson Respirometer or a Warburg Apparatus). The respiration of single cells has been measured with Cartesian divers.

These systems lend themselves primarily to fluid systems with a gas intermediate, an ideal arrangement for isolated organelles in suspension. It also works well for cells in suspension (algae, bacteria, yeast, tissue culture) and for moist tissue slices (leaf disks, liver slices).

Because of the nature of gas volume measurements, the primary difficulty with these techniques is control over temperature and atmospheric pressure. For simple procedures (such as the measure of gas evolved from an elodea leaf in a test tube) these are usually ignored or some type of control is attempted (water baths and heat shields for the lights). For accurate measurement on the cell level more extensive control must be had.

The Warburg Manometer was developed as a system to maintain constant temperature and volume, thus measuring changes in gas exchange by changes in pressure. It is extremely accurate, but it also is tedious and requires a good deal of mathematical corrections (made easier, of course, by using a computer). The Gilson Respirometer keeps constant temperature and pressure and monitors the change in gas exchange by changes in volume. To its advantage, the volume can be read directly and thus much of the mathematical corrections needed for the Warburg Manometer are eliminated. The constant pressure respirometer suffers in accuracy when compared to the constant volume manometer of Warburg, but it is sufficient for nearly all cell physiology purposes. Its inherent ability for easy automation has made it a standard for most cell labs. By contrast, the manometer remains the tool of choice for physical chemists.

The Gilson Constant Pressure Respirometer

The reaction flask consists of a main reaction chamber, a side arm, a center well and connections to a micrometer and a second chamber. The reaction is started by pouring the contents of the sidearm (usually a substrate) into the main vessel (containing the main reactants). The center well contains any materials which are accessory to the reaction (such as KOH to absorb CO_2). Changes in volume within the main vessel are altered through the use of a micrometer/piston system, and the micrometer is usually designed to read directly in microliters of gas exchanged. Interpretation of what gas has been exchanged is a function of the experiment design.

Exercise 8.7 - Respiration of Mitochondria

LEVEL III

Materials

- Gilson (or Warburg) Respirometer at 37° C
- Mitochondria suspension from Exercise 8.4
- Krebs Phosphate Ringers (KPR)
- 10% (w/v) Glucose
- 0.39% (w/v) Sodium azide

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- 18.4 mg% (w/v) Dinitrophenol (DNP)
- 6.64% (w/v) Sodium Malonate
- 10% (w/v) KOH

Procedure

1. When you enter the lab, check that the reference chambers of the Gilson respirometer are filled with the appropriate solution (usually formaldehyde). Turn on the instrument and equilibrate the temperature.
2. Line up fourteen reaction vessels and ensure that each is clean, free of cracks and has all the necessary springs (4 each), stoppers and vent tubes handy.
3. Use a micropipette to carefully place 0.2 ml of 10% KOH in the center well of each. It is extremely important that no KOH spill into the main reaction vessel,as this will destroy the mitochondria placed in the vessel.
4. Place 2.0 ml of KPR in the main vessel of flasks #1 and #2.
5. Place 0.5 ml of KPR in the main vessel of flasks #3 and #4.
6. Place 0.2 ml of KPR in the main vessel of flasks #5 through #12.
7. Place 0.1 ml of KPR in the main vessel of flasks #13 and #14.
8. Place 1.0 ml of mitochondria suspension in the main vessels of flasks #3 through #14.
9. Add 0.5 ml of glucose to the side arm of flasks #3 through #12.
10. Finally, add the following to the main vessels of the indicated flasks:

#5 and #6	0.3 ml of glucose
#7 and #8	0.3 ml of sodium azide
#9 and #10	0.3 ml of DNP
#11 and #12	0.3 ml of sodium malonate

11. Cut fourteen 2 cm squares from filter paper and make small fans out of each. Place one piece each into the center well. Doing this will increase the surface area available for CO_2 absorption by the KOH.
12. Carefully attach the sidearm stopper (or closed vent tube) to the flasks. Attach the flasks to the manometers and secure with springs. Lower the flasks into the water bath.
13. Allow all of the reaction flasks to temperature equilibrate for 5 minutes. During this time, set all of the micrometers to a reading of 500.
14. Add the contents of the sidearms to the main vessels by lifting the entire manometer assembly from the water bath and gently tipping the flask.

    BE CAREFUL NOT TO SPILL ANY CONTENTS INTO OR OUT OF THE CENTER WELL.

15. Place all of the flasks back into the water bath and close all valves of the respirometer. This is the zero point for your data - record the time.
16. Turn on the shaking motor and adjust the speed to obtain a gentle swirl of the flask contents.
17. After 10 minutes, adjust the manometer fluid to the original starting line by turning the appropriate

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micrometer. Record the micrometer readings for each of the fourteen flasks.

18. Repeat the micrometer readings at 10 minute intervals until a stable slope is obtained for the respirometers. This will generally be within 40-60 minutes from the zero point. Record all readings.
19. For each reading, subtract the original value of 500. Average the readings obtained in flasks #1 and #2 and subtract this average from the averages of #3/#4, #5/#6, #7/#8, #9/#10, #11/#12 and #13/#14.
20. Plot the change in gas volume (oxygen consumption) for each of the replicate conditions. 10

Notes

Tubes 1 and 2 are thermobarometers. Any changes in gas volume within these tubes reflects changes in the temperature and/or barometric pressure. Tubes 3/4 and 5/6 represent all of the conditions for oxidative phosphorylation (respiration). Tubes 7/8 are inhibited by azide which uncouples the electron transport system. Tubes 9/10 are inhibited by the presence of DNP which effects glucose transport as well as substrate entry into the electron transport system. Sodium malonate in tubes 11/12 is a competitive inhibitor of succinic dehydrogenase within the Krebs cycle. It will not halt the reactions of the ETS, and will only slow them down if glucose is in low concentration relative to the malonate. Finally, tubes 13/14 represent any endogenous reactions of the mitochondria in the sucrose isolation media.

Technically, glucose is not metabolized by the mitochondria. It must be altered by cytoplasmic enzymes to form pyruvate before entering the mitochondria. The isolation procedure used in mitochondrial preparation is important. If the mitochondria are "purified" the measurements will not work. Using a rough homogenate in sucrose will maintain enough enzymes for glycolysis to occur. The experiment could be altered by using pyruvic acid rather than the glucose. Glucose is used in the traditional experiment.

Record the fourteen manometer readings every 10 minutes for at least one hour. Average each pair of readings and substract the reading for 13/14 from each of the averages for 3/4, 5/6, 7/8, 9/10 and 11/12. Plot the corrected values against time, using a linear regression plot for each.

Flask #/Time	10	20	30	40	50
1					
2					
3					

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4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					

Exercise 8.8 - Photosynthesis by Respirometry

LEVEL III

Table 8.1 Characteristics of isolated chloroplasts

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Chloro-plast type	Description	Preparation method	Appearance under phase contrast microscopy	Envelope	Rate of CO_2 fixation (CO_2 /mg chlorophyll/hr)	Exogenous substrate penetration and requirements	Electron transport and photophosphorylation capacity
A	Complete chloroplasts	Rapid, in isotonic or hypertonic sugar, one centrifugation	Outer mobile jacket present. Bright and highly reflecting. Grana not obvious.	Intact	50 - 250	NADP and ferricyanide do not penetrate. Slow uptake of ATP, ADP and Pi.	Presumed to be unimpaired. (ATP/2e approaching 2.0 when assayed in hypotonic medium: good photosynthetic control)
B	Unbroken chloroplasts	In isotonic or hypertonic sugar or salt, with 2 or 3 centrifugations	Bright and highly reflecting. Smooth outline. Grana not obvious. (Class I)	Morphologi-cally but not functionally intact	<3	NADP, ferricyanide and ADP penetrate. Ferredoxin may not be necessary to add for NADP reduction.	Good ATP/2e (greater than 1.0 and often aproaching 2.0 when assayed in hypotonic medium). Good photosynthetic control.
C	Broken chloroplasts	Vigorous in isotonic sugar or salt	Not bright in appearance. 2-3 times larger than types A and B. Grana conspicuous. (Class II)	Broken and usually lost in prepara-tion (stroma also lost)	Little or none	NADP, ferricyanide and ADP penetrate. Ferredoxin needs to be added for NADP reduction	ATP/2e >1.0 or <1.0 depending on isolation and assay conditions. Some photosynthetic control
D	Free-lamellar chloroplasts	Osmotic shock of type A chloroplasts immediately followed by return to isotonic medium	----	Lost	High rates if carbon pathway intermediates and chloroplast extract added	----	Good photosynthetic control
E	Chloroplast	Resuspend	----	Lost	None	Ferredoxin	Higher rates of

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	fragments	chloroplasts in hypotonic medium				needs to be added for NADP reduction	electron transport and lower rates of photophosphorylation than types B and C. ATP/2e <1.0. No photosynthetic control
F	Sub-chloroplast particles	By sonication or detergent treatment or French press	----	Lost	None	Ferredoxin and plastocyanin (and some reductase needs to be added for NADP reduction)	Photophosphorylation (cyclic only) low or absent. Limited electron transport when electron donors added

Materials

- Gilson respirometer equipped with photoflood lamps
- Chloroplast suspension 11
- Chloroplast suspension buffer (suspension solution from Exercise 8.1)
- 0.1 M $NaHCO_3$
- Inhibitor 1 (1 x 10^{-4} DCMU)
- Inhibitor 2 (5 x 10^{-7} DCMU)

Procedure

1. In general, use the procedure listed in Exercise 8.7 for mitochondria respiration, except that the reactions are run at 25° C and a bank of photoflood lights must be used to ensure photosynthesis.
2. Set up the reactions vessels in the following manner:

Tube	Side arm	Main Vessel
1-2		2.0 ml Chloroplast buffer
3-4	0.5 ml $NaHCO_3$	0.5 ml buffer 1.0 ml Chloroplast Suspension
5-6	0.8 ml $NaHCO_3$	0.2 ml buffer 1.0 ml Chloroplast Suspension

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7-8	0.5 ml $NaHCO_3$ 0.3 ml Sodium Azide	0.2 ml buffer 1.0 ml Chloroplast Suspension
9-10	0.5 ml $NaHCO_3$ 0.3 ml Inhibitor 1	0.2 ml buffer 1.0 Chloroplast Suspension
11-12	0.5 ml $NaHCO_3$ 0.3 ml Inhibitor 2	0.2 ml buffer 1.0 ml Chloroplast Suspension
13-14	0.5 ml $NaHCO_3$	0.5 ml buffer 1.0 ml Boiled Chloroplasts

3. Measure the amount of oxygen evolved every 10 minutes until a steady rate is reached. Plot results and compare the effects of the selected inhibitors.

Endnotes

---

1. Chloroplasts are extremely light labile. They should be kept cool and in reduced light at all times, except when actually being used for analysis of photosynthesis. Once diluted, the chloroplasts will be reasonably unstable and you should work as rapidly as possible while keeping all reactants as cool as possible.
2. Be careful to not confuse the pigment CHLOROPHYLL with the organelle CHLOROPLAST.
3. DCMU is a powerful electron acceptor and will inhibit System II electron transfers in intact chloroplasts.
4. May substitute 10 mM Tetrazolium Blue @ 580 nm, 20 mM Methyl Red @ 440 nm, or .001 M C_2 ,C_3 ,C_5 ,C_6 -Tetramethyl-p-pheynlenediamine (DAD) if System I is to be specifically measured. DAD will reverse the uncoupling of System I induced by DCMU.
5. Sunlight is best, but photofloods will do. If photoflood lights are used, a heat filter must be used. This can be accomplished by placing a small aquarium full of water between the light source and the beaker containing your chloroplast suspensions.
6. Fresh cauliflower can be substituted if the homogenization is done with a mortar and pestle. There will be some plastid contamination, but it should not affect respiration measurements with normal room light exposure. Weigh cauliflower and begin with step 3. There will be significantly fewer mitochondria isolated per gram of tissue than with liver.
7. May be omitted. It works to protect the mitochondria from rapid degradation.
8. Prepare just before use. A stock solution of 0.33% may be stored frozen in an amber bottle. Thaw and dilute just before use.
9. Oxidation of p-phenylenediamine oxalate (PPDO) changes the dye from its colorless form (reduced) to purple (oxidized).
10. This is easier to do if the data are entered directly into a computer spreadsheet program. Refer to Appendix D.
11. The isolation of intact chloroplasts is a significant factor in the success of this exercise. Many of the structures and functions of chloroplasts are altered during the extraction process. Refer to Table 8.1.

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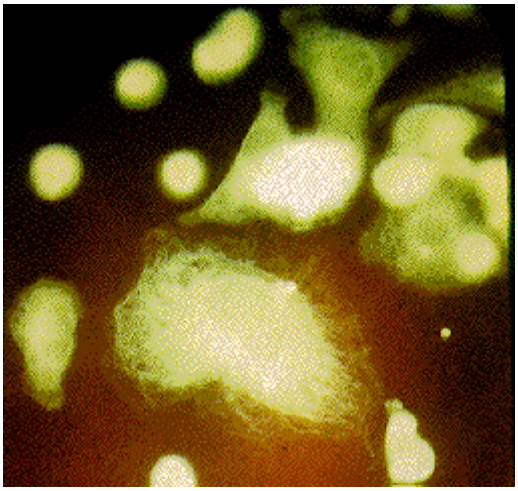
Chapter 9: Tubules\Filaments

Introduction

Figure 9.1 Macrophage treated with anti-tubulin



Original photo compliments of Dr. John Lammert, Gustavus Adolphus College

Table 9.1 Intermediate filaments and matrix components

Filament Type	Molecular weight in K daltons	Protein	Tissue
Tonafilaments	40 - 68	Cytokeratins	Keratinizing and non-keratinizing epithelia; mesothelium
Glial filaments	51	Glial fibrillary acidic protein	Astroglia, Bergmann glia
Desmin filaments	53	Desmin	Skeletal, cardiac and smooth muscle
Vimentin filaments	53.5	Vimentin	Mesenchyme derived cells and tissues
Neurofilaments	68,160,200	Neurofilaments triplet proteins	Neurons of the central and peripheral nervous system

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Matrix Component	Distribution	Features
Interstitial collagen type 1	skin, bone, dentin, tendon, cornea	presence of α2 chain, less than 10 hydroxylysines/chain, 0.1% carbohydrate
Interstitial collagen type 2	cartilage, vitreous body, notochord	1% carbohydrate, greater than 10 hydroxylysines/chain
Interstitial collagen type 3	as type 1, prominent in fetal skin, arteries, amniotic membrane, not in bone or tendon	presence of cysteine, high levels of hydroxyproline,glycine, histidine
Basement membrane collagen type 4	basement membranes (lamina densa)	greater than 20 hydroxylysines/chain, high 3-hydroxyproline, low alanine and arginine
Collagen type 5	associated with membraneous structures	slightly larger than α 21(1), 0.3% 3-hydroxyproline
Fibronectin	loose connective tissue matrix, basement membranes, body fluids	
Laminin	basement membranes (lamina rara)	ordered polypeptide structures (α-chain, β-sheet, disulfide knot)
Tropoelastin-Elastin	elastic fibres	high content of alanine, valine, glycine contains hydroxyproline, dross-linking
Microfibrillin		
Proteoglycans		
Glycosaminoglycans, sulfated proteoglycans, hyaluronic acid		

Cell shape is determined by microscaffolding created by microtubules and microfilaments. Internally, these tubules and filaments make up the cytoskeleton. Externally they are the components of the extracellular matrix (glycocalyx and plant cell wall).

Microtubules have a diameter of about 24 nm and are usually found in groups of 13 protofilaments. The basic structural subunit is a dimer of two similar proteins, αand βtubulin, each with a molecular weight of 55,000 daltons. Tubulin and several larger microtubule-associated proteins (MAPs) interact in the presence of GTP and Mg^{++} to form hollow tubes known as protofilaments which can stretch for long distances across the cytoplasm. The MAPs function to anchor the tubulin protofilaments together into larger structures (centrioles, basal bodies, flagella) and to attach the protofilaments to other structures within the cell (nuclear envelope, chromosomes) as well as to other filamentous molecules (actin, intermediate filaments).

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Polymerized tubules are also important in cell division,motility, communication and differentiation (morphogenesis). They are believed to be important elements of neural cell function and glandular secretion.

Within a cell, microtubule integrity is affected by temperature. Microtubules are relatively stable at physiological temperatures, but below 10° C they spontaneously depolymerize into constituent subunits, namely tubulin and the microtubule-associated proteins (MAP's). When the cells are returned to 37° C, microtubules are reformed in about 15 minutes (providing that sufficient GTP and Mg^{++} are present).

Microtubules

One of the several methods currently used to isolate microtubules takes advantage of the temperature-dependent stability of the microtubule proteins. Polymerized microtubules maintain their structural organization at physiological temperatures, but rapidly depolymerize below 10° C. A crude extract of tissue containing large numbers of microtubules is warmed to 37° C in a buffer containing GTP, EGTA (a chelating agent that binds calcium ions) and Mg^{++}. Microtubules will form in approximately 15 minutes.

As the proteins polymerize into tubules, the viscosity of the solution will increase. Increased viscosity can be monitored directly with a viscometer, or indirectly by a change in sedimentation coefficient or by an alteration in the turbidity of the solution. Turbidity is the easiest alteration to monitor, since it can be measured with a UV spectrophotometer.

After the formation of the microtubules, a pellet enriched in microtubules may be prepared by centrifugation of the viscous solution at warm temperatures (10° C). The pellet is then depolymerized by incubation at 4° C in buffer, once again yielding a solution of tubulin, with a slight decrease in yield, but an increase in the purity. The supernatant containing both tubulin and microtubule-associated protein is again warmed for microtubule polymerization. Several cycles of this temperature dependent, assembly/disassembly procedure results in highly pure preparations of microtubules.

The polymerized microtubule structure can be examined by transmission electron microscopy for structural integrity. The microtubules are negatively stained and dried directly onto a coated EM grid for observation. Alternatively, they can be pelleted, embedded and sectioned for routine TEM analysis. Since the tubulin and MAPs are protein, antibodies can be made against the molecules. These can be used either for EM analysis or for immunofluorescent analysis at the light microscope level.

Microtubule assembly can be inhibited by several chemical agents. Colchicine or vincristine depolymerize microtubules, and/or prevent their polymerization from the monomer tubulin. Colchicine is an alkaloid extracted from the plant Colchicum sativum. Colcemid, a synthetic derivative, is commonly used in tissue cultures to halt mitotic cells at metaphase. When colcemid is removed, spindle fibers will form and the cell will divide. If the colcemid treatment is prolonged, the cell will not divide and will result in polyploid formation..

Immunofluorescent Analysis

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Cells fixed at 37° C in their microtubular structure. To visualize the organization of the microtubules, plasma membranes are solubilized and made permeable to anti-tubulin antibodies. If the antibody has previously been bonded to a fluorescent dye, the organization of microtubules within the cell can be observed with a fluorescent microscope.

Microfilaments

One of the most studied forms of molecular architecture associated with cellular function has been the relationship of thick and thin filaments of the striated muscle of vertebrates. The thin actin filaments (6-8 nm diameter) are interposed between the thick myosin filaments (18 nm diameter) and these in turn are often associated with intermediate filaments (about 10 nm diameter).

Figure 9.1 presents a schematic of the relationship of these filaments in a typical striated muscle while Figure 9.2 gives both light and TEM views.

In smooth muscle, the connections are not as clearly defined, but the presence of the three types of filaments can be observed. In smooth muscle, the intermediate filaments are integrated into the thin filaments.

Imtermediate Filaments

Any analysis of microfilaments would be incomplete without some study of the host of filaments collectively referred to as intermediate filaments.

There are five types of intermediate filaments: tonofilaments (keratins or cytokeratins), neurofilaments, glial filaments, desmin filaments and vimentin filaments. For further details, refer to Table 9.1.

GAGs

There are a host of filamentous and globular proteins on the surface of cells which are composed of both proteins and carbohydrates. Older terminology referred to these as glycoproteins if there was more protein than carbohydrate and mucopolysaccharides if there was more carbohydrate. Today, the common term is Glycos Amino Glycans or GAGs. Examples of GAGs are collagenous and non-collagenous glycoproteins, elastin, sulphated proteoglycans (heparin) and complexes with hyaluronic acid.

GAGs are involved in the formation of the "pericellular" matrices of developing cells. These matrices are important for cell-cell contact in early embryogenesis and differentiation, and in the formation of extracellular basement membranes. There is evidence that they are involved in information processing of RNA within the cell as well as their structural function. Receptor mediated endocytosis is also dependent upon these surface molecules as is normal receptor activities of hormones, neurohormones and the activation of cAMP.

Exercise 9.1 - Fluorescent Analysis of Microtubules

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LEVEL I

Materials

- Projection slides of tubulin fluorescence1

Procedure

1. Draw the cells represented in Figure 9.1. These are photographs of macrophages treated with a fluorescent anti-tubulin antibody.
2. Describe the shape and general appearance of the cells observed in the first three slides. How does the organization of the microtubules affect cell shape?
3. Identify a place in the cell from which the microtubules appear to extend. This is the microtubule-organizing center. Label it in the appropriate drawings.
4. The cells in slide 4 were exposed to colchicine for 30 minutes and then prepared as above. What do you predict will happen to the microtubules after cell treatment with colchicine? Was this prediction fulfilled? Draw a colchicine-treated cell.
5. The cells in slide 5 were placed on ice for 30 minutes and then prepared as above. What do you predict will happen to the microtubules after incubation of the cells in the cold? Is the microtubule diameter larger or smaller than the resolving power of the light microscope? How can microtubules be visualized with immunofluorescence if the tubules are less than the resolution of the light microscope?

Exercise 9.2 - Actin& Myosin-Microscopic Observation of Muscle

LEVEL I

Materials

- Glycerinated rabbit muscle fibers
- 5 mM ATP in 0.01 M Tris-HCl buffer, pH 7.6 plus 0.05 M KCl
- 0.001 M $CaCl_2$
- Prepared slides of striated muscles (longitudinal sections)
- Phase contrast microscope, slides, coverslips

Procedure

1. Obtain a prepared slide of striated muscle cells. Draw the structure of an individual cell, paying particular attention to the striations. Measure the distance between successive bands of the muscle and indicate these on the drawing.
2. Compare the image seen in the light microscopic to that in Figure 9.2.
3. Tease a small fiber from the glycerinated rabbit muscle and place in on a microscope slide. Place a coverslip gently over the muscle fiber and examine with a phase contrast microscope.
4. Measure the distance between the bands and compare to the distance found in the prepared slide.
5. Gently lift off the coverslip (or use a second fiber) and add a drop of ATP and a drop of $CaCl_2$. Return the coverslip and immediately monitor the cells through the microscope. Once again measure the distance between the bands.

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Optional

To observe the contraction of the muscle, carefully add the ATP and Ca++ to the edge of the coverslip and allow it to diffuse under the coverslip. You may also time the contraction process with a stopwatch. Finally, variations in the ion concentration as well as alternative ions can be added to other muscle fibers to test the effects of ions on muscle contraction.

Exercise 9.3 - Histology of Extracellular Matrix

LEVEL I

Materials

- Prepared slides of

Intestine	Basement Membranes
Loose connective tissue	Fibronectin
Lamina rara of epidermal tissues	Laminin
Skin and/or bone	Collagen Type I
Hyaline cartilage	Collagen Type II
Amniotic membrane	Collagen Type III
Elastic Fibers	Elastin

- Microscope

Procedure

1. Components of the extra cellular matrix are too small to be observed directly with a light microscope, but their presence can be observed in the structures they form. The list of slides above includes the basic intermediate filament type associated with each tissue structure. Examine each of the slides, paying particular attention to the location and structure of any extracellular matrices.
2. Draw each of the tissues. Label the location and type of matrix component expected in each tissue.

Exercise 9.4 - Isolation of Microtubules (Bovine Brain)

LEVEL II

Materials

- Freshly removed bovine brain 2
- Wire sieve (tea strainer)
- Microtubule buffer (MT buffer)
    - 0.1 M MES (2-(N-Morphilino)ethanesulfonic acid)

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    - 1 mM EGTA (Ethylene Glycol-bis(β-aminoethyl Ether) N,N,N',N'-tetraacetic acid)
    - 0.5 mM $MgCl_2$
    - Adjust pH to 6.4
- 8 M Glycerol in MT buffer
- Virtis homogenizer (or equivalent)
- Refrigerated centrifuge
- GTP
- Bradford protein assay

Procedure [3]

1. Remove the meninges and peripheral blood vessels from the brain. Puree the brain by pushing it through a wire sieve and directly into a chilled beaker.
2. Homogenize the cerebral hemispheres of the brain at 4° C in a tissue homogenizer capable of homogenizing with minimal sheer force. [4] Use the maximum speed allowable for your homogenizer for 10 seconds. Place the brain hemispheres in the homogenizer with microtubule buffer at a ratio of 0.5 ml of MT buffer to 1 g of wet weight of brain tissue.
3. Centrifuge the crude brain homogenate at 19,600 xg for 30 minutes at 4° C to remove connective tissue and cellular debris.
4. Decant the supernatant and recentrifuge the supernatant at 27,000 xg for 45 minutes at 4° C for further clarification.
5. Collect 10 ml of the supernatant and determine the protein content of your sample using the Bradford protein assay (Appendix G). Use bovine serum albumin or lysozyme for establishing a standard curve.
6. Decant the remainder of the crude supernatant into a chilled beaker, and add an equal volume of 8 M glycerol in MT buffer.
7. Add dry GTP (MW 523) to the crude supernatant to make a 1.0 mM final concentration of GTP and incubate the mixture at 37° C for 30 minutes.
8. Centrifuge the mixture at 100,000 xg for 60 minutes at 25° C. It is crucial that the temperature be maintained at 25° C. At a lower temperature the microtubules will not polymerize (thus no pellet); and at a higher temperature the tubulin may be degraded.
9. Remove the pellet and resuspend in 40 ml of cold MT buffer. Incubate for 30 minutes at 4° C. Occasional homogenization in a ground glass homogenizer will facilitate depolymerization of the microtubules.

   For good tubulin polymerization, a series of polymerizations and depolymerizations, with subsequent centrifuge collections is required. Steps 7 through 9 should be repeated a minimum of 3 times.

10. With each successive polymerization with GTP and subsequent collection of the precipitated microtubules, repeat the protein determination on each sample.

Exercise 9.5 - Viscosity & Polymeriztion of Microtubules

LEVEL II

Materials

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- Tubulin (Brain extract from Exercise 9.4)
- GTP
- ATP
- Viscometer
- Waterbath or incubator at 37° C

Procedure

1. Compute the amount of GTP (M.W. 523) needed to make a final concentration 0.5 mM GTP when added to 40 ml. of brain extract in microtubule buffer. Likewise, compute the amount of dry ATP (M.W 507) needed to make a final concentration of 0.5 mM ATP.
2. With the brain extract in an ice bath, add both the GTP and the ATP (swirl to dissolve) and immediately transfer the mixture to a water bath at 37° C.
3. Use a viscometer to measure the viscosity at 3 minute intervals for a period of 30 minutes, or until change is no longer observed.

   A viscometer is a device which makes use of capillary flow. Insert a glass viscometer into a water bath at the appropriate temperature (37° C for polymerization) and add a 3.0 ml sample to the viscometer. Apply suction or pressure as appropriate to the viscometer to pull the sample into the expanded sample chamber. Release the suction (or pressure) and measure the time it takes for the sample to move from the upper mark on the viscometer to the lower mark.

   Use sucrose solutions of known viscosity to calibrate the viscometer.

4. Plot the change in viscosity with time of incubation.

Notes

Tubulin and MAP's will polymerize in the presence of Mg^{++} and GTP. The tubulin extract is placed in a buffer that supplies all of the necessary ingredients, except GTP. Upon the addition of GTP, the tubulin will begin to polymerize and can be monitored.

Exercise 9.6- Polymerization Induced Changes in Absorbance

LEVEL II

Materials

- UV spectrophotometer
- Water baths set at 10° C and 37° C
- Tubulin extract from Exercise 9.5
- MT buffer, ATP and GTP

Procedure

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1. Turn on the spectrophotometer and adjust the wavelength to 350 nm. Adjust the spectrophotometer to read absorbance, using microtubule buffer as a blank.
2. Starting with the tubulin extract in the water bath and equilibrated to 10° C, add ATP and GTP as in steps 1 and 2 of Exercise 9.5. Immediately measure the absorbance of the solution. Speed is important! Place the sample still in the spectrophotometer cuvette back into the 10° C waterbath.
3. At 30 second intervals remove the cuvette, wipe it dry and measure the absorbance. Immediately replace the sample and cuvette back in the 10° C waterbath. Repeat this for a total period of 3 minutes.
4. After the 3 minute interval, place the cuvette and its contents in the 37° C waterbath.
5. Continue to read the absorbance at 30 second intervals, returning the sample to the 37° C waterbath between readings.
6. When the absorbance value stabilizes (about 5 minutes), replace the sample and cuvette back in the 10° C bath and continue to measure the absorbance at 30 second intervals.
7. Plot absorbance against time, indicating points of temperature change.

Notes

You may monitor the kinetics of tubule formation by continuously measuring the absorbance of the solution at 350 nm, provided a temperature controlled sample holder is available. Preferably, the sample temperature should be able to be rapidly changed from 10° C to 37° C and back. This can be accomplished via a temperature-controlled kinetic spectrophotometer (such as a Beckman DU Kinetic spectrophotometer, or equivalent). Alternatively, a water jacketed sample cuvette can be engineered with some simple tubing and valves to switch the water flow through the jacket.

Exercise 9.7 - TEM Visualization of Microtubules

LEVEL II

Materials

- Coated grid for TEM
- 0.1 M ammonium acetate
- 5% ethanol saturated uranyl acetate
- Transmission electron microscope

Procedure

1. At the conclusion of a 37° C incubation from Exercise 9.6, remove one drop (approximately 20 microliters) of the extract, place it on a collodion or formvar and carbon-coated EM grid, and allow it to remain for about 20-30 seconds. 6
2. Rinse the grid carefully with 0.1 M ammonium acetate, and float the grid upside down on a drop of 5% uranyl acetate for 15 minutes. Rinse the grid with water, blot all excess fluids from the grid, and allow it to air dry.
3. Examine the stained grid in a transmission electron microscope.
4. Draw or photograph your observations -- calculate all appropriate dimensions (length, diameter) of the formed tubules.

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Exercise 9.8 - SDS Gel Electrophoresis of Tubulin\MAPs

LEVEL II

Materials

- Stock Acrylamide: (30%T:0.8%C)
  - 30% by weight of acrylamide
  - 0.8% by weight of N,N'-bis-methylene acrylamide
- Separation Gel (Final Concentrations)
  - 10% acrylamide (1:3 dilution of stock)
  - 0.375 M Tris-HCl (pH 8.8)
  - 0.1 % SDS
- Stacking Gel (Final Concentrations)
  - 3 % acrylamide (1:10 dilution of stock)
  - 0.125 M Tris-HCl (pH 6.8)
  - 0.1% SDS
- Electrode Buffer
  - 0.025 M Tris
  - 0.192 M Glycine
  - 0.1% SDS
  - Adjust pH to 8.3
- 0.2-0.3 ml samples of brain extract, and containing
  - 200 micrograms protein
  - 0.0625 M Tris-HCl (pH 6.8)
  - 2% SDS
  - 10% glycerol
  - 5% β-mercaptoethanol
  - 0.001 % bromophenol blue
- TEMED
- 10% (w/v) Ammonium persulphate
- 50% and 7% (w/v) TCA (trichloroacetic acid)
- 0.1% (w/v) Coomassie brilliant blue in 50% TCA

Procedure

1. Prepare 15 cm glass tubes with 6 mm internal diameter. Polymerize a 10 cm separation gel and a 1 cm stacking gel within the tubes by the addition of TEMED and ammonium persulfate as directed in Chapter Four.
2. Assemble all the equipment and place 200 µl of sample in one tube. Set up a second gel containing 200 µl of protein molecular weight standards.
3. Electrophoresis is carried out at 3 mA per gel until the bromophenol blue dye reaches the bottom of the tube (approximately 7 hours).
4. Fix the gels overnight in 50% TCA.
5. Stain with 0.1% Coomassie brilliant blue (fresh in 50% TCA) for 1 hour at 37° C.

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6. Diffusion-destain with repeated washing in 7% TCA.
7. Scan the gels or photographically analyze them.
8. Determine the molecular weights of the proteins (tubulin and MAP's).

Exercise 9.9 - Isolation of Actin and Myosin Filaments

LEVEL III

Materials

- Relaxing Solution
  - 0.1 M KCl
  - 0.001 M $MgCl_2$
  - 5 mM ATP
  - 0.016 M NaH_2PO_4
  - Na_2HPO_4
  - Adjust pH to 7.3
- 0.05 M Sodium phosphate buffer, pH 7.0
- 0.001 M EDTA (Ethylene diamine tetra acetic acid)
- Blender
- Preparative centrifuge
- Materials for TEM fixation, embedding and observation

Procedure 7

1. Obtain fresh chicken gizzards 8 and dissect the lateral muscles free from all attachments. Place the muscles on crushed ice and then grind them in a standard worm- drive meat grinder. Small samples can be pushed through a hand press, if desired.
2. Weigh the tissue and add an equal volume of cold 0.05 M Sodium phosphate buffer, pH 7.0 with 0.001 M EDTA. Blend this mix in a standard blender at low speed and pour the slurry into a large beaker.
3. Upon settling, the muscle fragments will settle on top of the underlying connective tissue. Separate the two by decanting, and concentrate by low speed centrifugation.
4. Suspend aliquots of blended muscle in two volumes of relaxing solution and homogenize in a blender at high speed for 30 seconds.
5. Centrifuge the homogenate at 500 xg to remove membraneous organelles and whole cells. The myofilaments are preferentially localized in the middle, clear solution of the centrifuge tube.
6. Collect the middle layer and recentrifuge at 40,000 xg to pellet the myofilaments. Wash and gently resuspend. This will rid the preparation of soluble proteins.
7. Prepare a small sample of the middle layer from Step 5 for EM observation, following the procedure outlined above for tubulin (Exercise 9.7).

Exercise 9.10 - Further Studies

LEVEL III

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Additional analysis of the interaction of tubulin and MAPs can be made following the separation of the proteins. This can be accomplished by phophocellulose chromatography, as described in Aamodt and Williams. 9 Once separated, the polymerization can be monitored with varying ratios of tubulin to MAP, and in the presence of various polymerization inhibitors (colchicine, vincristine).

Other studies could be made of isolated cellular structures, such as flagella, cilia, or even isolated spindle fibers. The latter can be isolated in quantity from sea urchin embryos, as outlined by K.A. Suprenant. 10

Collect egg and sperm by injection of KCL, and combine the two for fertilization. As the embryo's reach metaphase (80-85 minutes at 18° C), they are collected, washed free of salt water and homogenized in a low pH PIPES buffer containing Mg^{++} , EGTA and Triton X-100 detergent. The spindles are pelleted at 1000 Xg (clinical centrifuge), washed several times and monitored with polarization microscopy. Since the spindles are highly birefringent, they can be studied without the use of a TEM. They can be observed fairly readily with standard enhanced phase contrast optics or stained for bright field optics.

The sea urchin also serves as a ready source of flagella (sperm) and cilia (developing pluteus). Virtually all of the early work on tubulin polymerization was accomplished with this model system.

Endnotes

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1. It is convenient to use permanent photographic slides, since the microscope preparations are unstable (flurorescence causes bleaching of the dye). Several companies produce fluorescent antibodies to tubulin and also supply protocols for producing the slides. Refer to Exercise 2.7 for further details.
2. Available from slaughter houses, or any animal brain may be substituted. It is important that the brain be freshly removed from a sacrificied animal, however.
3. From Shelanski, M.L., F. Gaskin and C.R. Cantor. 1973. "Microtubule assembly in the absence of added nucleotides." *PROC. NATL. ACAD. SCI. USA* 70:765-768. For a modified Weissenberg Technique and Sephadex Purification of tubulin cf. G.C. Na and S.N. Timasheff "Physical Properties of Purified Calf Brain Tubulin" in Methods in Enzymology, Vol. 85, p. 393-408. 1982.
4. A household blender should only be used as a last resort.
5. Crude estimation of an increase in viscosity can be had by observing the rate of escape for trapped air bubbles as the solution is swirled. Viscosmeters are available commercially at reasonable prices. Alternatively, a pipette can be utilized. Mix a reasonable sample of sucrose concentrations (look up viscosities in handbook of physical constants) and simply calibrate the viscometer by measuring the time it takes to deliver 3 ml from the filled pipette.
6. Alternatively, the pellets containin microtubules can be collected, fixed, embedded and sectioned for EM examination. This will require several days, however, and will yield sectioned views of the tubules, rather than intact whole tubules.
7. Cooke, P. "Preparation of Isolated Thick and Thin Filaments" in Methods in Enzymology, Vol. 85, pp 277-284., 1982.

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8. It is possible to isolate the individual fibers from various sources, but avian gizzards have been most often used for preparation of thick and thin filaments. They can be obtained from chicken slaughterhouses in mass quantity, and are virtually pure smooth muscle.
9. Aamodt, E.J. and R.C. Williams, Jr. 1984. "Microtubule-associated proteins connect microtubules and neurofilaments in vitro." *Biochemistry* 23:6023-6031.
10. In *Methods in Cell Biology* Vol. 27: Echinoderm Gametes and Embryos (T.E. Schroeder, ed.). Academic Press, Orlando, 1986. pp. 189-216.

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Chapter 10: Chromosomes

Introduction

The interphase chromosomes of eucaryotic cells are complex molecular structures composed primarily of a DNA core and a protein matrix complexed into a long thread-like structure. This basic chromosome thread is then coiled through several layers of organization and ultimately gives rise to a structure that can be visualized with a light microscope.

Chemically, the interphase nucleus is composed of a substance known as "chromatin," which is further subdivided into euchromatin and heterochromatin. The distinction between these subdivisions is based on quantitative distribution of the basic chromosome fiber, with a higher concentration found in heterochromatin. Heterochromatin, therefore, will stain more intensely than euchromatin, since the fiber is packed tighter within a given volume.

Proteins extracted from chromatin have been classified as either basic or acidic in nature. The basic proteins are referred to as "histones" and the acidic as "non-histone proteins". Histones play an integral part in the structural integrity of a eucaryotic chromosome. They are organized into specific complexes, known as nucleosomes, and around which the DNA molecule is coiled. Acidic proteins within the nucleus compose many of the DNA replication and RNA transcription enzymes and regulatory molecules. They vary in size from small peptides of a few amino acids to large duplicase and replicase enzymes (respectively DNA and RNA polymerases).

Transcription of DNA on the chromosome fiber results in the presence of a host of RNA species found within the nucleus of the cell. When the RNA is transcribed from the "nucleolar organizer" region of a genome and complexed with ribosomal proteins, granules are formed which collectively produce a "nucleolus," visible at the light microscope level of resolution. When transcribed from other portions of the genome, the RNA is either in the form of pre-transfer RNA, or heterogeneous nuclear RNA (hnRNA). The precursor tRNA must be methylated and altered before becoming functional within the cytoplasm, and the hnRNA will also be significantly modified to form functional mRNA in the cytoplasm.

Thus, a chemical analysis of chromosomes will yield DNA, RNA and both acidic and basic proteins. [1] It is possible to extract these compounds from an interphase nucleus (i.e. from chromatin) or to physically isolate metaphase chromosomes and then extract the components. For the former, the nuclear envelope will be a contaminating factor, as will the nucleolus. For isolated chromosomes, many of the regulatory molecules may be lost, since the chromosomes are essentially non- functional during this condensation period.

Exercise 10.1 - Polytene Chromosomes of Dipterans

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LEVEL I

Materials

- Prepared slides of *Drosophila* (fruit fly) salivary gland chromosomes
- Genetic map of polytene chromosome bands

Procedure

1. Examine the slides for the presence of bands. Select a single chromosome spread demonstrating all four chromosomes and draw the complete structure.
2. Label each of the four chromosomes of the fruit fly, as well as the chromocenter of the connected chromosomes.
3. Compare your drawings to the genetic map for *Drosophila*.

Notes

The glands of dipterans (flies) have a useful characteristic for analysis of gene location on chromosomes. During their mitotic division, the normal division of the chromosomes is aborted and the replicated chromosomes remain as an integral unit. The chromosome content thus increases geometrically and produces "giant" polytene chromosomes. The chromosomes remain attached at a point where the centromeres fuse, at the chromocenter.1 This is clearly observed in the chromosomes of the fruit fly salivary gland tissue. The fruit fly chromosomes are ideal specimens since they are in a near constant state of prophase and are incapable of further division. Because they have been extensively analyzed for their genetic composition, co-linear maps of genes within genetic linkage groups have been produced and correlated with the physical location of a band on the chromosomes.

Exercise 10.2-Salivary Gland Preparation (Squash technigue)

LEVEL I

Materials

- Fruit fly larva (wild type and tandem duplication mutants)
- Ringers insect saline
- Fine forceps and probe
- Aceto-orcein
- Dissecting and regular microscopes
- Slides, coverslips
- Small dish of melted paraffin and paintbrush 2

Procedure

1. Select a third instar larva, for which the cuticle has not yet hardened, from a wild-type culture of *Drosophila*. Place it into a drop of Ringer's saline solution on a slide.

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2. Place the slide on the stage of a dissecting microscope and view the larva with low power. Grasp the anterior of the larva with a fine point forceps and pin down the posterior portion with a probe. Gently pull the head off and discard the tail of the larva.
3. Locate the salivary glands and their attached fat bodies. The glands are semitransparent and attached by ducts to the digestive system. The fat bodies are white and opaque. Tease away the fat bodies and discard.
4. Place a drop of aceto-orcein on the slide next to the Ringer's and move the salivary glands into the stain. Blot away any excess Ringer's.
5. Place a coverslip over the preparation and allow it to stand for 1-3 minutes (it will take a few trials to obtain properly stained chromosomes). Gently squash the gland preparation in the following manner:
    - Place the slide between several layers of paper toweling.
    - Place your thumb on the top of the towel immediately over the coverslip and gently roll your thumb while exerting a small amount of pressure (as though you were making a finger print). Do not move your thumb back and forth. One gentle roll is sufficient.
    - Remove the slide from the towels, and seal the edges of the coverslip by using a paint brush dipped in melted paraffin.
6. Examine the slide with the microscope and diagram the banding patterns that are observed.
7. Compare your squash preparation to that of the prepared slides examined in Exercise 10.1.
8. Repeat the squash technique using larva from a genetic variant known to be the result of a deletion and/or tandem duplication. Determine the location of the deleted or duplicated bands on the chromosomes.

Exercise 10.3 - Extraction of Chromatin

LEVEL II

Materials

- Bovine or porcine brain
- 0.25 M Sucrose containing 0.0033 M calcium acetate
- 2.0 M Sucrose with 0.0033 M calcium acetate
- 0.075 M NaCl with 0.024 M EDTA, pH 8.0
- Tris-HCl buffer, pH 8.0 with the following molarities

    0.05 M, 0.002 M, 0.0004 M

- TCA (Trichloroacetic acid)
- Tissue homogenizer
- Cheesecloth
- Refrigerated preparative centrifuge
- Bradford or Lowry protein assay
- UV spectrophotometer (Optional)

Procedure 3

1. Homogenize approximately 30 gms of bovine or porcine cerebellar tissue in a teflon-glass homogenizer

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in 9 volumes of cold 0.25 M sucrose containing 0.0033 M Calcium Acetate.

2. Filter the resulting brei through several layers of cheesecloth and obtain crude nuclear pellets by centrifuging at 3,500 xg for 20 minutes.
3. Resuspend the nuclear pellet in 80 ml of cold 0.25 Sucrose containing 0.0033 M Calcium Acetate. [4]
4. Obtain three cellulose nitrate centrifuge tubes and place 25 ml. aliquots of the resuspended nuclear pellet in each. Carefully pipette 5.0 ml of 2.0 M Sucrose-0.0033 M Calcium Acetate into the bottom of each tube. Insert a pipette with the 2.0 M sucrose through the suspended nuclei and allow the viscous sucrose to layer on the bottom of the tube. Centrifuge the tubes at 40,000 xg for 60 minutes.
5. Using the resulting nuclear pellet is just above the dense sucrose layer. It is used to extract chromatin proteins. Carefully remove the supernatant above the pellet with a pipet. Then, insert the pipet through the nuclear layer and remove the bottom sucrose layer. The nuclear pellet will remain in the tube. Resuspend the pellet in 40 ml of 0.075 M NaCl-0.024 M EDTA, pH 8.0 and centrifuge at 7700 xg for 15 minutes.
6. Remove and discard the supernatant, resuspend the pellet once again in 40 ml of 0.075 M NaCl-0.024 M EDTA, pH 8.0 and centrifuge again at 7700 xg for 15 minutes. Repeat this process one more time.
7. Resuspend the nuclear pellet in 40 ml of 0.05 M Tris-HCl, pH 8.0 and centrifuge at 7,700 xg for 10 minutes.
8. Repeat step 7 to thoroughly wash the nuclei and then wash 2x each in 0.01 M Tris pH 8.0, 0.002 M Tris pH 8.0 and 0.0004 M Tris pH 8.0.
9. Resuspend the final washed nuclear pellet in ice cold distilled water to a final volume of 100 ml and allow to swell overnight at 4° C. Gently stir the mixture on the following day. This solution is the pure chromatin to be used for subsequent analysis.
10. Determine the purity of the chromatin sample within the nuclear pellet using one of the following:
    - Determine the protein concentration by Lowry or Bradford procedure.
    - Measure the optical absorbance at 230 nm (UV). The absorbance of a 1 mg/ml concentration of pea bud histone at 230 nm equals 3.5. OD units. The absorbance follows the Beer-Lambert law, and is linear with histone concentration. Since it is merely an optical reading, the sample is not destroyed in the measurement.
    - Measure the turbidity of the solution. Add trichloroacetic acid to a final concentration of 1.1 M and wait exactly 15 minutes. Read the OD_{400}. A 10 ug/ml solution of pea bud histone has an OD = 0.083 at 400 nm. This technique is excellent for readings between 0 and 0.15 OD. The TCA precipitates some proteins and thus this procedure is more specific to histones than B. It can also be performed without a UV spectrophotometer.
    - Measure by non-destructive fluorometry. Histones can be detected by an excitation wavelength of 280 nm and a fluorescence measurement at 308 nm. Non-histones can be detected in the same sample by excitation at 290 nm and measurement at 345 nm. Of course, this procedure requires the use of a fluorescence spectrophotometer.

Notes

The extraction of chromatin proteins starts with the isolation of a good nuclear fraction. Nuclear pellets and chromatin should be extracted one day before the laboratory period. If DNA, RNA, and both Histone and Non-Histone proteins are to be separated, begin the procedure approximately three working days prior to lab.

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Exercise 10.4 - Chromatin Electrophoresis

LEVEL II

Materials

- 14 M Urea
- 6 M NaCl
- 0.05 M and 0.9 M Acetic acid
- Dialysis tubing
- Electrophoresis apparatus
- Prepared gels
- 10 M urea-0.9 N acetic acid-0.5 M β-mercaptoethanol
- 0.25% Coomasie blue

Procedure

1. To the chromatin suspension from Exercise 10.3, add concentrated urea and concentrated NaCl separately to yield a final concentration of 7 M urea and 3 M NaCl.
2. Centrifuge the clear solution at 85,500 xg for 48 hours at 4° C to pellet extracted DNA.
3. Collect the supernatant and dialyze it against 0.05 M acetic acid (three changes, 6 liters each at 4° C). Remove the dialyzed protein solution and lyophilize it to dryness.
4. Meanwhile, set up a standard polyacrylamide gel (Exercise 4.1), using 15% acrylamide (15%T:5%C) in 2.5 M urea and 0.9 M acetic acid. Set up the gel in the electrophoresis unit and run the gel at 2 mA/gel for 2 hours with no sample, using 0.9 M acetic acid for the running buffer.
5. Dissolve the lyophilized protein from step 3 in 10 M urea-0.9 N acetic acid-0.5 M β-mercaptoethanol (to a final concentration of 500 micrograms protein per 100 µl of buffer) and incubate at room temperature for 12-14 hours prior to the next step.
6. Apply 20 µl samples of the redissolved protein extract to 0.6 x 8.0 cm polyacrylamide prepared as in step 4.
7. The gels are run against 0.9 M acetic acid in both upper and lower baths for approximately 3 hours at 100 V.
8. Stain the gels for 1 hour in Coomasie blue, rinse with water, destain and store in 7% acetic acid.
9. If densitometry measurements are made, 5 µg of pea bud fraction IIa protein has a density of 1.360 density units x mm with a 95% confidence limit of 10%. By comparison, the density value can be used to quantitate the concentration of protein fractions in µg of your sample.

Exercise 10.5 - Extraction and Electrophoresis of Histones

LEVEL II

Table 10.1 Properties of chromatin

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Morphotype	Activated chromatin Euchromatin	Non-activated chromatin Facultative and obligate heterochromatin, euchromatin
Structural organization	Less condensed, unfolding of functional domains (2040 kbp) exhibit	Highly condensed
DNA methylation (CG sites)	DNase I sensitive sites m*v*-sites unmethylated	m*v*-sites methylated
Nucleosomes	DNase I sensitive	DNase I resistant
Histones	H1-deprivated; core histones highly acetylated	H1-enriched; association with special H1 isofores, e.g., H5; H2A/H2B under-acetylated; eventually H2A modified by ubiquitin
HMG 14/17	Present	Absent
HMG 1/2	Present	Absent
Transcription RNAP/RNP	Presence of RNAP and RNP depends on the actual transcription state	No

---

Materials

- Saline Citrate (1/10 SSC)
- 1.0 N H_2SO_4
- Refrigerated preparative centrifuge
- Absolute ethanol
- β-mercaptoethanol
- 0.01 M Sodium phosphate buffer, pH 7.0 + 1% (w/v) SDS + 0.1% (v/v) β-mercaptoethanol
- 10% acrylamide gels (10%T:5%C) with 0.1% (w/v) SDS
- 7% (w/v) Acetic acid
- 0.25% Coomasie blue

Procedure [5]

1. Dissolve crude chromatin in cold dilute saline citrate (0.015 M NaCl + 0.001 M Sodium Citrate) to a final DNA concentration of 500 µg/ml.
2. Stir the solution on ice and slowly add 1/4 volume of cold 1.0 N H_2SO_4. Continue stirring for 30 minutes.

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3. Centrifuge the suspension at 12,000 xg for 20 minutes at 4° C. Save the supernatant. For maximum yield, break up the pellet, resuspend in fresh, cold 0.4 N H_2SO_4, re-extract, centrifuge, and add the resulting supernatant to the first.
4. Add 4 volumes of cold absolute ethanol to the supernatant and store for 24 hours at -10° C to precipitate the histone-sulfates.
5. Collect the precipitate by centrifugation at 2,000 xg for 30 minutes.
6. Decant as much of the alcohol as possible, and resuspend the pellet in cold absolute ethanol.
7. Centrifuge at 10,000 xg for 15 minutes.
8. Collect the pellet and freeze dry for later analysis.
   To continue with the electrophoresis, carefully weigh the histone protein sample and dissolve in 0.01 M sodium phosphate buffer, with a pH 7.0 and containing 1% sodium dodecyl sulfate and 0.1% β-mercaptoethanol; final volume should contain approximately 300 µg of protein in 100 µl of buffer.
9. Prepare the electrophoresis chamber with a 10% acrylamide gel with 0.1% SDS.
10. Add separately 25 µl of the dissolved protein and 25 µl of protein standards to:

    50 µl of 0.1% SDS, 0.1% β-mercaptoethanol in Buffer
    5 µl of β-mercaptoethanol
    1 µl of 0.1% bromophenol blue in water

11. Mix thoroughly and apply the histone extract and protein standards to separate wells of the electrophoresis gel.
12. Separate the proteins in the anode direction (Anionic system). 6
    - The addition of SDS anions to the proteins results in negatively charged proteins which will separate according to molecular weight.
    - Electrophoresis is carried out in the standard manner following the basic steps given in Chapter Four. The buffer utilized is that of Laemmli, 7 0.025 M Tris-0.192 Glycine and 0.1% SDS, pH 8.3.
    - Proteins are separated by a current of 3-4 mA per gel until the bromophenol marker reaches the bottom of the tube (about 7 hours at 3 mA, and 4 hours at 4 mA).
13. Stain the gels with 0.25% Coomasie Blue for 2 hours.
14. Destain and store in 7% acetic acid.
15. Scan the gels and determine the molecular weights of each component.

Notes

Preparation of a total histone fraction from nuclei is normally accomplished by extraction with a dilute acid or a high molarity salt solution. The acidic extraction removes histones from DNA and non-histones immediately, while the dissociation of chromatin in salt solutions will require further purification. In either event, the histones themselves are subdivided into five major types, designated as H1, H2, H3, H4 and H5. H2 dissociates into two peptides, which are thus designated as H2A and H2B. The classification of histones is based on their electrophoretic mobility.

Non-histone proteins can also be extracted and separated by electrophoresis. Whereas histones have only 5 major types, non- histones are extremely heterogeneous and up to 500 different proteins have been identified from one cell type, while the major proteins comprise less than 20 types.

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The extraction of chromatin DNA was possible with the 7 M urea - 3 M NaCl extraction performed in Exercise 10.4. Further analysis of DNA will be undertaken as part of a later lab exercise (on Transcription), and the DNA sample from this lab may be kept lyophilized and frozen until that time.

For our current needs it is sufficient to note that the genes are composed of DNA, and that various specific regions of the DNA/Genetic information can be physically isolated to a specific locus on a chromosome. This in turn is readily observed and correlated with banding patterns, such as those in the fruit fly poltytene chromosomes.

Exercise 10.6 - Karyotype Analysis

LEVEL II

Table 10.2 Human chromosome dimensions

Chromosome #	Relative length	Centromere index
1	9.08	48.0
2	8.45	38.1
3	7.06	45.9
4	6.55	27.6
5	6.13	27.4
6	5.84	37.7
7	5.28	37.3
X	5.80	36.9
8	4.96	35.9
9	4.83	33.3
10	4.68	31.2
11	4.63	35.6

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12	4.46	30.9
13	3.64	14.8
14	3.55	15.5
15	3.36	14.9
16	3.23	40.6
17	3.15	31.4
18	2.76	26.1
19	2.52	42.9
20	2.33	44.6
21	1.83	25.7
22	1.68	25.0
Y	1.96	16.3

Relative length in percentage of Total Haploid Autosome Length.
Centromere index as length of short arm divided by total chromosome length X 100.

Human Karyotype Form

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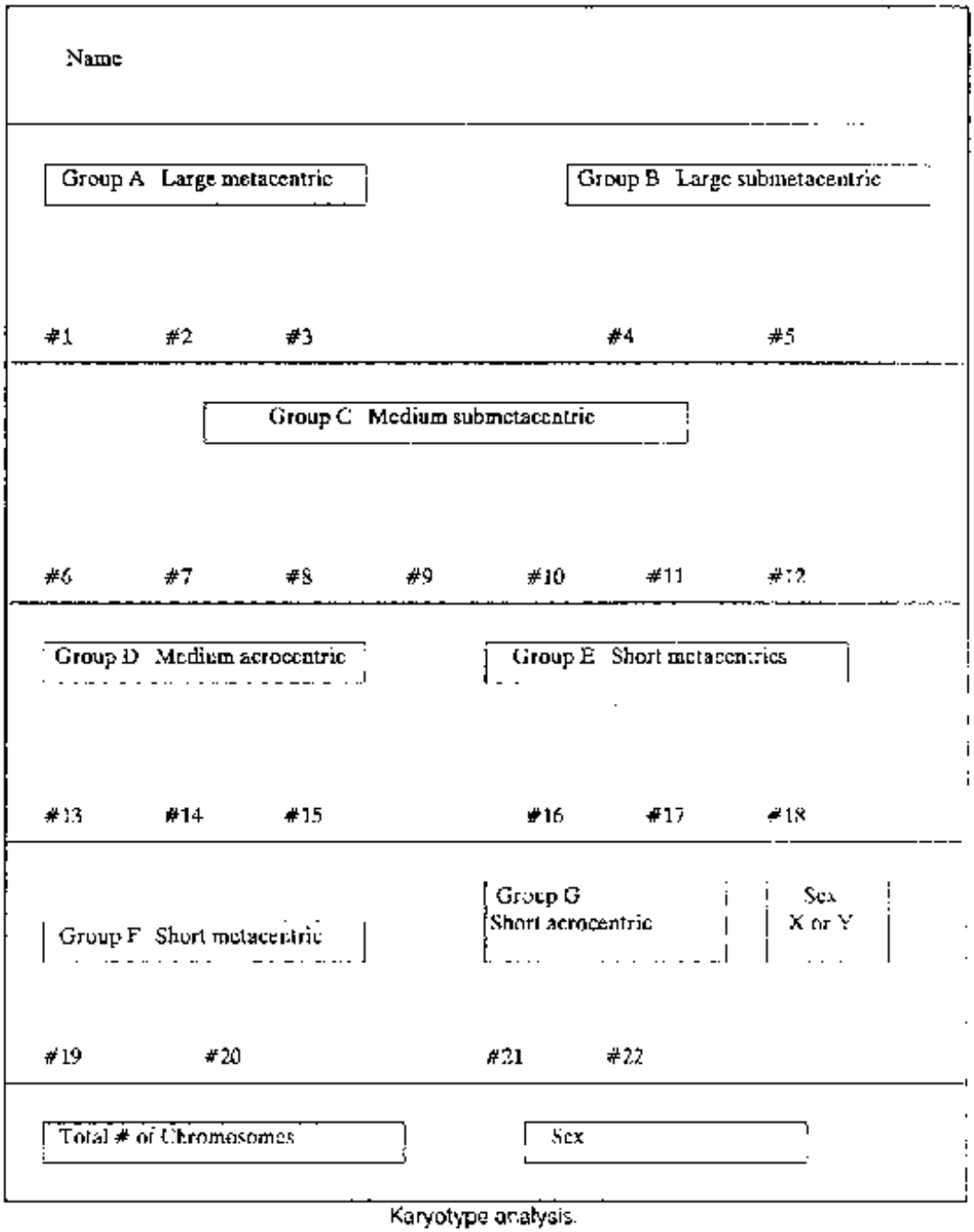

Karyotype analysis.

Materials

- Fresh venous blood [8]
- Heparinized syringes
- Eagle's spinner modified media with PHA
- Culture flasks
- Tissue culture grade incubator at 37° C
- 10 µ g/ml Colcemid
- Clinical centrifuge and tubes

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- 0.075 M KCl
- Absolute Methanol and glacial acetic acid (3:1 Mixture, prepared fresh)
- Dry ice
- Slides, coverslips and permount
- Alkaline solution for G-banding
- Saline-Citrate for G-banding
- Ethanol (70% and 95% (v/v))
- Giemsa stain

Procedure 9

1. Draw 5 ml of venous blood into a sterile syringe containing 0.5 ml of sodium heparin (1000 units/ml). The blood may be collected in a heparinized Vacutainer, 10 and transferred to a syringe.
2. Bend a clean, covered 18 gauge needle to a 45° angle and place on the syringe. Invert the syringe (needle pointing up, plunger down), and stand it on end for 1-1/2 to 2 hours at room temperature.
   During this time the erythrocytes settle by gravity, leaving approximately 4 ml of leukocyte-rich plasma on the top, and a white buffy coat of leukocytes in the middle.
3. Carefully tip the syringe (do not invert) and slowly expel the leukocyte-rich plasma and the buffy coat into a sterile tissue culture flask containing 8 ml of Eagle's spinner modified media supplemented with 0.1 ml of phytohemagllutin (PHA).

   BE EXTREMELY CAREFUL NOT TO DISRUPT THE RED BLOOD CELLS IN THE BOTTOM OF THE SYRINGE. RBC'S WILL INHIBIT GROWTH OF THE LEUKOCYTES.

4. Incubate the culture for 66-72 hours at 37° C. Gently agitate the culture once or twice daily during the incubation period.
5. Add 0.1 ml of colcemid (10 micrograms/ml) to the culture flasks and incubate for an additional 2 hours.11
6. Transfer the colcemid-treated cells to a 15 ml centrifuge tube and centrifuge at 225 xg for 10 minutes.
7. Aspirate and discard all but 0.5 ml of the supernatant. Gently tap the bottom of the centrifuge tube to resuspend the cells in the remaining 0.5 ml of culture media.
8. Add 10 ml of 0.075 M KCl, dropwise at first, and then with gentle agitation to the centrifuge tube. Gently mix with each drop.

   START TIMING THE NEXT STEP IMMEDIATELY WITH THE FIRST DROP OF KCL.

9. Let the cells stand **EXACTLY** 6 minutes in the hypotonic KCl.

   THE HYPOTONIC SOLUTION SHOULD NOT BE IN CONTACT WITH THE CELLS IN EXCESS OF 15 MINUTES FROM THE TIME IT IS ADDED.

10. Centrifuge the cells at 225 xg for 6 minutes. Aspirate the KCl and discard all but 0.5 ml of the supernatant. Gently resuspend the cells in this small volume of fluid.
11. Add 10 ml freshly prepared fixative dropwise at first and then with gentle agitation. Gentle and continuous agitation is important at this step to prevent clumping of the cells. If the cells were not

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properly resuspended in step 10, the cells will clump beyond any further use.

12. Allow the cells to stand in fixative at room temperature for 30 minutes.
13. Centrifuge at 200 xg for 5 minutes and remove all but 0.5 ml of supernatant. Resuspend the cells in fresh fixative.
14. Wash the cells twice more in 10 ml volumes of fixative. Add the fixative slowly, recentrifuge, and aspirate the fixative as previously directed.

THE FIXED, PELLETED CELLS MAY BE STORED FOR SEVERAL WEEKS AT 4° C.

15. Resuspend the pellet of cells in just enough fixative to give a slightly turbid appearance.
16. Prop a piece of dry ice against the side of a styrofoam container and lace a clean slide onto the dry ice to chill the slide.

DRY ICE WILL CAUSE FROST BITE. HANDLE WITH TONGS ONLY.

Use a **siliconized** pasteur pipette to draw up a few drops of the suspended cells and drop the cells onto the surface of the chilled slide. Spreading of the chromosomes may be enhanced by dropping the cell suspension from a height of at least 12 inches. As soon as the cells strike the slide, blow hard on the slide to rapidly spread the cells. [12]

FOR BEST RESULTS ALLOW ONLY ONE DROP PER SLIDE.

17. Remove the slides from the dry ice and allow them to air dry. Perform the desired banding and/or staining procedures.

Procedure = Preparation of chromosomes for karyotype analysis can be performed in a number of ways and each will yield differing pieces of information. The chromosomes may be stained with aceto- orcein, feulgen or a basophilic dye such as toluidine blue or methylene blue if only the general morphology is desired.

Procedure = If more detail is desired, the chromosomes can be treated with various enzymes in combination with stains to yield banding patterns on each chromosome. These techniques have become common place and will yield far more diagnostic information than giemsa stain alone (the most commonly used process). A band is an area of a chromosome which is clearly distinct from its neighboring area, but may be lighter or darker than its neighboring region. The standard methods of banding are the Q, G, R, and C banding techniques. These are defined as follows:

- Q-banding
    1. Quinacrine stain
    2. Fluorescence microscopy
- G-banding
    1. Giemsa stain
    2. Additional Conditions
        - a. Heat hydrolysis
        - b. Trypsin treatment

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        - c. Giemsa at pH 9.0
    - R-banding
        1. Giemsa or acridine orange
        2. Negative bands of Q and G reversed
        3. Heat hydrolysis in buffered salt
    - C-banding
        1. Giemsa stain
        2. Pretreatment with BaOH or NaOH followed by heat and salt.

The following directions are for a G-banding: 13

- Treat fixed and flamed slides in alkaline solution, room temperature for 30 seconds.
- Rinse in saline-citrate solution, 3 changes for 5-10 minutes each.
- Incubate in saline-citrate solution, 65° C for 60-72 hours.
- Treat with 3 changes of 70% ethanol and 3 changes of 95% ethanol (3 minutes) each.
- Air dry.
- Stain in buffered Giemsa for 5 minutes.
- Rinse briefly in distilled water.
- Air dry and mount.

18. Photograph appropriate spreads and produce 8 X 10 high contrast photographs of your chromosome spreads.
19. Cut each chromosome from the photograph and arrange the chromosomes according to size and position of the centromere.
20. Use Table 10.2 to identify the specific chromosomes.
21. Tape or glue each chromosome to the form supplied for this purpose.

Exercise 10.7 - *In Situ* Hybridization

LEVEL III

A modern approach to the specific location of genes on chromosomes is a technique for the hybridization of DNA and RNA "in situ." With this procedure, specific radioactive RNA or DNA (known as probes) can be isolated (or synthesized "in vitro") and then annealed to chromosomes which have been treated in such a manner that their basic double stranded DNA has been "melted" or dissociated.

In theory, and fortunately in practice, when the DNA is allowed to re-anneal, the probe competes for the binding, but only where it mirrors a complimentary sequence. Thus, RNA will attach to the location on the chromosome where the code for its production is to be found. DNA will anneal to either RNA which is still attached to a chromosome, or to the complimentary sequence DNA strand within the chromosome. Since the probe is radioactive, it can be localized via autoradiographic techniques.

Finally, it is possible to produce an RNA probe that is synthesized directly from repetitive sequences of DNA, such as that found within the nucleolar organizer region of the genome. This

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RNA is known as cRNA (for copied RNA) and is a convenient source of a probe for localizing the nucleolar organizer gene within the nucleus, or on a specific chromosome.

The use of in situ hybridization begins with good cytological preparations of the cells to be studied, and the preparation of pure radioactive probes for the analysis. The details depend upon whether the hybridization is between DNA (probe) and DNA (chromosome), DNA (probe) and RNA (chromosome), or between RNA (probe) and DNA (chromosome).

Preparation of the Probe: 14

Produce radioactive RNA by incubating the cells to be measured in the presence of ^{3}H-uracil, a specific precursor to RNA. Subsequent to this incubation, extract rRNA from the sample and purify through differential centrifugation, column chromatography or electrophoresis. Dissolve the radioactive RNA probe in 4X Saline-Citrate containing 50% formamide to yield a sample that has 50,000 to 100,000 counts per minute, per 30 microliter sample, as determined with a scintillation counter. Add the formamide is added to prevent the aggregation of RNA.

Preparation of the Slides:

Fix the materials to be studied in either 95% ethanol or in 3:1 methanol:water, attach to pre-subbed slides (as squashes for chromosomes) and air dry.

Hybridization

Place the air dried slides into a moist chamber, usually a disposable petri dish containing filter paper and carefully place 30 microliters of RNA probe in 4X SSC-50% formamide onto the sample.

Carefully add a cover slip (as in the preparation of a wet mount), place the top on the container and place in an incubator at 37° C for 6-12 hours.

Washing:

1. Pick up the slides and dip into 2X SSC so that the coverglass falls off.
2. Place the slides in a coplin jar containing 2X SSC for 15 minutes at room temperature.
3. Transfer the slides to a treatment with RNase (50 microgram/ml RNase A, 100 units/ml RNase T1 in 2X SSC) at 37° C for 1 hour.
4. Wash twice in 2X SSC, 15 minutes each.
5. Wash twice in 70% ethanol, twice in 95% ethanol and air dry.

Autoradiography:

Add photographic emulsions to the slides and after a suitable exposure period, develop the slides, counterstain and add cover slips.

Analyze the slides by determining the location of the radioactive probe on the chromosomes or within the nuclei.

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Endnotes

1. Occasionally, lipids are found within nuclei. It is usually associated with degeneration of the nuclear structure, or with neoplastic alterations of the nucleus.
2. This is a standard means of sealing wet mounts. Melted paraffin is highly flammable, however, and should never be melted with an open flame. The author has found that the slides can be sealed just as well with a coat of nail polish.
3. From: L.M.J. Shaw and R.C.C. Huang. "A Description of Two Procedures Which Avoid the Use of Extreme pH Conditions for the Resolution of Components Isolated from Chromatins Prepared from Pig Cerebellar and Pituitary Nuclei" *Biochemistry* 9:23 1970. pp 4530-4542.
4. A sample may be taken and fixed for light and electron microscope observation at a later time.
5. Bonner et al.
6. K. Weber and M. Osborn. 1969. "The Reliability of Molecular Weight Determinations by Dodecyl Sulfate Polyacrylamide Gel Electrophoresis" *J. Biol. Chem.* 244:16, 4406-4412.
7. U.K Laemmli. 1970. "Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4" *Nature* Vol. 227. pp. 680-685.
8. The procedure is based on one performed at the Centersfor Disease Control, Atlanta, GA on human blood samples. Virtually any animal blood can be substituted, or if cells in culture are available, log phase cultures can be substituted and the process begun with step 5. Blood can be drawn at a local clinic directly into heparanized tubes, and should be disposed of properly.
9. This procedure can be handled as a Level I exercise by purchasing photographs of chromosme spreads (Carolina Biological) and skipping to step 19.
10. Vacutainer Systems, Order #6481. Becton Dickinson, Rutherford, NJ 07070
11. If an inverted phase microscope is available, you can check that the cultures are in a log phase of growth before adding the colcemid.
12. The general process involves swelling of the cells in the hypotonic KCl treatment and dropping them like big water balloons onto the slide. The bigger "splash" they make on impact, the better the chromosomes will spread out as the nuclei burst on contact with the slide. Some investigators actually drop the cells from a distance of five feet. The author has found this to be challenging and fun, but of no real advantage. The chilled slide aids in sticking the chromosomes onto the slide. Siliconized pipettes are necessary to prevent the cells from adhering to the walls of the pipette during transfer. This is the most common reason for not obtaining good chromosome preparations.
13. If only morphology is to be studied, skip steps a-e and begin with step f, but increase the staining to 10-20 minutes.
14. Summarized from H. Macgregor and J. Varley. *Working with Animal Chromosomes*. John Wiley & Sons, Chichester, 1983. pp. 196-201.

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Chapter 11: Cell Cycles

Introduction

The onion root tip and the whitefish blastula remain as the standard introduction to the study of mitosis. The onion has easily observable chromosomes, and the whitefish has one of the clearest views of the spindle apparatus. The testis of the grasshopper and the developing zygote of the roundworm Ascaris are the traditional materials used for viewing the various stages of meiosis.

In a single longitudinal section of a grasshopper testis one can usually find all of the stages of meiotic development. The stages are also aligned from one pole of the testis. Few other meiotic samples are as convenient. For most material, meiosis occurs in a more randomly distributed pattern throughout the testis.

Ascaris is utilized to observe the final stages of development in eggs (oogenesis). The Ascaris egg lies dormant until fertilized. It then completes meiosis forming two polar bodies while the sperm nucleus awaits fushion with the female nucleus. When this phenomenon is coupled to the large abundance of eggs in the Ascaris body, it makes an ideal specimen for observing the events of fertilization, polar body formation, fusion of pronuclei and the subsequent division of the cell (cytokinesis).

Interphase G1-S-G2

The stages of mitosis were originally detailed after careful analysis of fixed cells. More recently, time lapse photography coupled with phase contrast microscopy has allowed us to visualize the process in its entirety, revealing a dynamic state of flux.

In early work, so much emphasis was placed on the movement of the chromosomes that the cell was considered to be "at rest" when not in mitosis. As significant as mitotic division is, it represents only a small fraction of the life span of a cell. None the less, you may still come across the term "resting phase" in some older texts. This term is rarely used today, and the term interphase is sufficient for all activities between two mitotic divisions. The cell is highly active during interphase and most of the metabolic and genetic functions of the cell are reduced during the physical division of the nuclear and cellular materials (mitosis).

Figure 11.1 presents our current view of a cell cycles. Note that interphase is divided into three sub-phases, G1, S and G2. The basis for this division is the synthesis of DNA. Note also that while the entire cycle may be as long as 24 hours, mitosis is normally less than one hour in length.

Because of the synthesis of DNA in interphase, the amount of DNA per nucleus is different depending on which sub phase of interphase the cell is in. DNA can be measured using the fuelgen reaction and a microscpectrophotometer. The basic amount of DNA in a haploid nucleus is given

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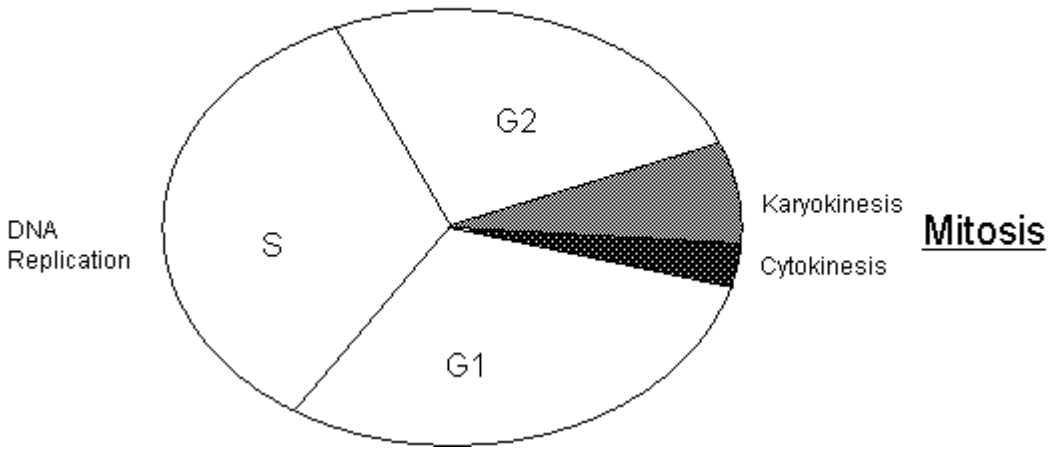
the value C. A diploid nucleus would be 2C. A triploid and tetraploid cell would be 3C and 4C respectively.

However, when nuclei are actually measured, diploid cells in interphase can be divided into three groups; some are 2C, some are 4C while a few are at intermediate values between 2C and 4C. The conclusion is that the genetic material (DNA) and presumably the chromosomes must duplicate. The period within interphase and during which DNA is synthesized is termed the S phase (for Synthesis). The period of interphase preceding the S phase is the G1 phase (for 1st Growth Phase), while the period subsequent to the S phase is the G2 phase (for the 2nd Growth Phase). During the G1 period, the cell is generally increasing in size and protein content. During S, the cell replicates the chromosomes and synthesizes DNA. During G2, it continues to increase in size, but also begins to build a significant pool of ATP and other high energy phosphates, which are believed to be a significant part of the triggering mechanism for the subsequent karyokinetic and cytokinetic events of mitosis.

Mitosis returns the cells to the 2C state. Meiosis reduces the amount of DNA even further, to 1C. Meanwhile the number of chromosomes (designated with the letter N) is also changing. For a diploid cell, the number of chromosomes is twice that of a haploid, or 2N. During mitosis, a diploid cell would go from one 2N cell to two 2N cells. Since the daughter cells have the same chromosome number as the parent, mitosis is also referred to as equational division. If a diploid (2N) cell undergoes meiosis, it will result in four haploid cells, each 1N. Thus, meiosis is also referred to as reductional division. Refer to Figure 11.2 for comparison of C and N values during division.

Figure 11.1 Cell cycle



It is possible to visualize the process of DNA synthesis within either nuclei or chromosomes by the incorporation of a radioactive precursor to DNA into cells and subsequent detection by

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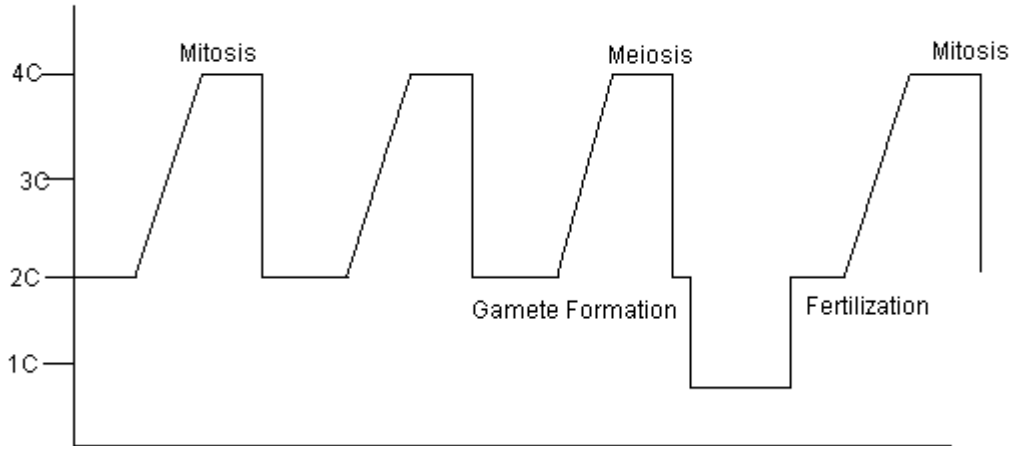
autoradiography. Incorporation of thymidine, a DNA precursor, will only occur during the S phase, and not during G1 nor G2. If a pulse (short period of exposure) of ^{3}H-thymidine is presented to cells, those that are in the S period will incorporate this radioactive substance, while all others will not. Careful application of the pulse will allow the timing of the S phase. By knowing the timing for the entire cycle (from mitosis to mitosis), one can deduce the G1 and G2 periods.

Figure 11.2 Amount of DNA present during division



Meiotic division differs from mitosis in that there are two division cycles instead on one. In the first cycle, interphase is the same as for mitosis. That is there is an S phase with corresponding G1 and G2. During the second interphase, however, no DNA synthesis occurs. Consequently there is no G1 or G2 in the second interphase. The result is that chromosomes are replicated prior to Meiosis, and do not replicate again during meiosis.

For the following details of Mitosis, refer to Figure 11.3 Stages of mitosis in onion root tip

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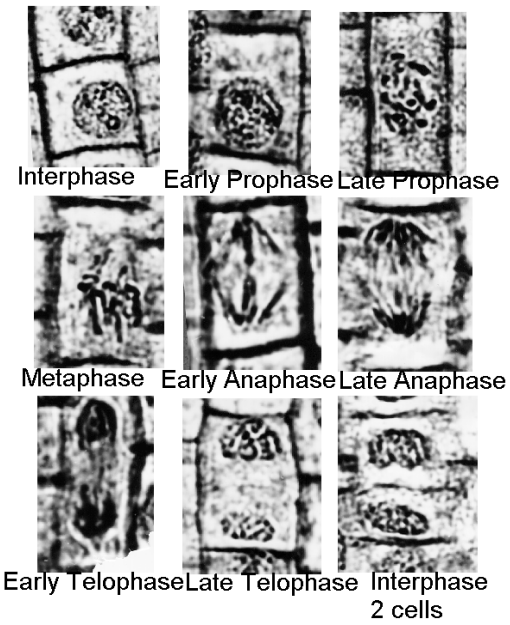

Prophase

The first phase of mitosis is marked by the early condensation of the chromosomes into visible structures. At first, the chromatids are barely visible, but as they continue to coil, the chromosomes become thicker and shorter. The nuclear envelope is still present during this stage, as are any nucleolar structures. The centrioles are moving to the poles of the cell and spindle fibers are just beginning to form.

Metaphase

During the middle phase of karyokinesis, the chromosomes line up in the center of the cell, and form a metaphase plate. Viewed on edge, the chromosomes appear to be aligned across the entire cell, but viewed from 90 Degrees they appear to be spread throughout the entire cell (visualize a plate from its edge or from above). Each chromosome has a clear primary constriction, the centromere, and attached to each is a definitive spindle fiber. The spindle apparatus is completely formed, and the centrioles have reached their respective poles. The nucleolus and the nuclear envelope have disappeared.

Anaphase

The movement phase begins precisely as the two halves of a chromosome, the chromatids, separate and begin moving to the opposite poles. The centromere will lead the way in this process, and the chromatids form a V with the centromeres pointing toward the respective poles.

Telophase

The last phase is identified by the aggregation of the chromatids (now known as chromosomes) at the respective poles. During this phase, the chromosomes uncoil, the nuclear envelope is resynthesized, the spindle apparatus is dismantled and the nucleolus begins to appear.

Meiosis

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For meiosis, the phases prophase, metaphase, anaphase and telophase are identified, but because there are two divisions, there are two sets. These are designated by Roman numberals; thus Prophase I, Metaphase I, Anaphase I, Telophase I, Interphase, Prophase II, Metaphase II, Anaphase II and Telophase II. Interphase is normally not designated with a Roman numberal. Because of the significance of the chromosome pairing which occurs in Prophase I, it is further subdivided into stages. For the following descriptions of meiosis, refer to the phases of Prophase I are named for the appearance of a thread-like structure, known as nema. Leptonema means "thin thread" and leptotene is the adjective applied to the term stage, i.e. proper terminology is the leptotene stage of prophase I. The word stage is often omitted.

Figure 11.4 Selected stages of meiosis in grasshopper testis

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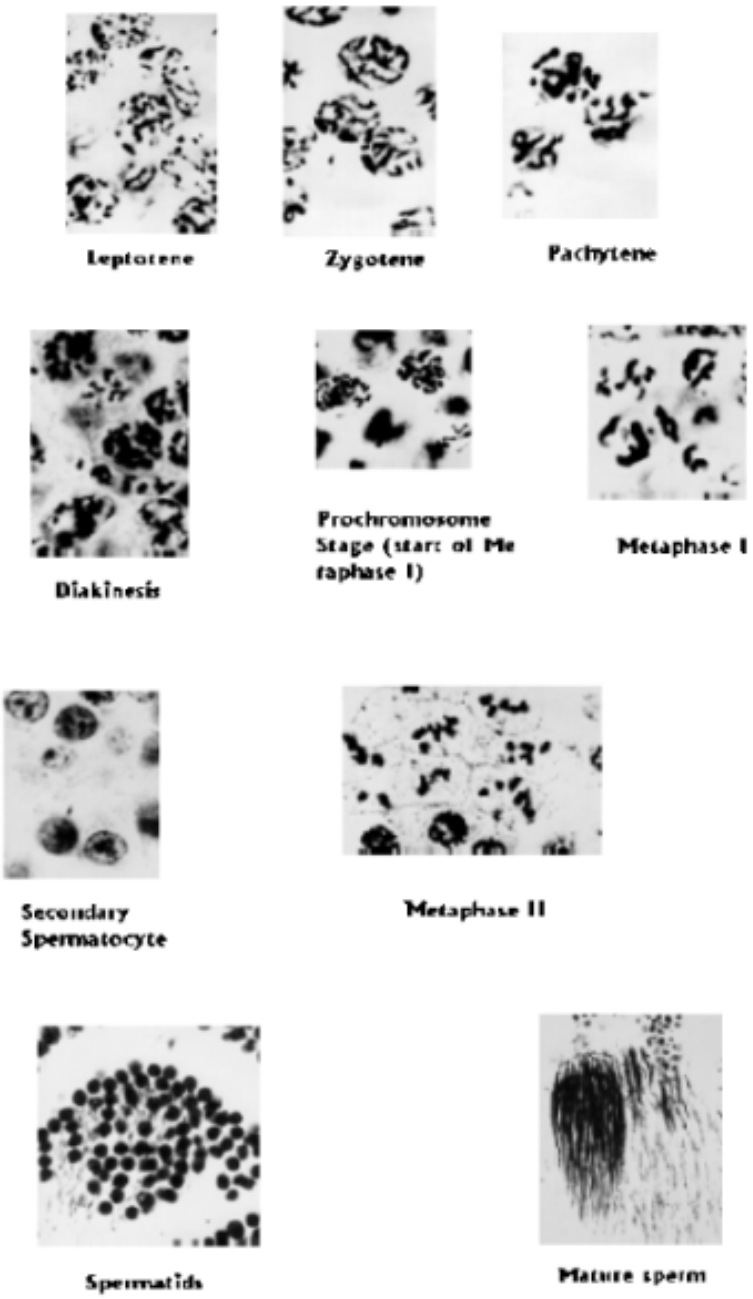

Prophase I: Leptotene [1]

This stage is marked by the first appearance of the chromosomes when the chromosomes are in their most extended form (except for during interphase). They appear to be a string with beads. The beads are known as chromomeres. The chromatids have already replicated prior to this phase, but typically, the replicated chromatids can not be observed during the leptotene stage.

Prophase I: Zygotene

Zygos means "yoked," and during this stage, the homologous chromosomes are seen as paired units. The chromosomes are shorter and thicker than in leptotene, and in some cells they remain

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attached to the nuclear envelope at the points near the aster. This gives rise to an image termed the "bouquet." This attachment is rare in invertebrates and absent in plants, where the chromosomes appear to be a tangled mass.

Prophase I: Pachytene

When the pairing of zygotene is complete, the chromosomes appear as "thick" strings, or pachynema. The chromsomes are about 1/4 the length they were in leptotene, and there are obviously two chromosomes, each with two chromatids in each bundle. The two chromosomes are referred to as a "bivalent," while the same structure viewed as four chromatids is known as a "tetrad."

Prophase I: Diplotene

This stage results as the gap between the two homologous chromosomes widens. The homologs have already paired during zygotene, recombined during pachytene and are now beginning to repel each other. During this stage, the chromosomes of some species uncoil somewhat, reversing the normal direction typical of prophase. As the chromosomes separate, they are observed to remain attached at points known as "chiasmata." These are believed to be the locations where genetic recombination of the genes has taken place.

Prophase I: Diakinesis

Prophase I ends as the homologs completely repel each other. The chromosomes will continue to coil tightly (reversing the slight uncoiling of the diplotene) and will reach their greatest state of contraction. As diakinesis progresses, chiasmata appear to move toward the ends of the chromosomes, a process known as "terminalization." Since this stage is the end of prophase, the nucleolus usually disappears, along with the nuclear envelope.

Metaphase I

The tetrads move toward the center and line up on a metaphase plate. The nuclear envelope completely disappears. As the tetrads align themselves in the middle of the cell, they attach to spindle fibers in a unique manner. The centromeres of a given homolog will attach to the spindles from only one pole.

Anaphase I

The unique event occurring at this phase is the separation of the homologs. In contrast to mitotic anaphase, the centromeres of a given homolog do not divide, and consequently each homolog moves toward opposite poles. This results in a halving of the number of chromosomes, and is the basis of the reduction division that characterizes meiosis.

Telophase I: Interphase

Telophase in meiosis is similar to that of mitosis, except that in many species, the chromosomes do not completely uncoil. If the chromosomes do uncoil and enter a brief interphase, there is no

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replication of the chromatids. Remember that the chromatids have already been replicated prior to Prophase I.

Prophase II - Telophase II

These phases are essentially identical in meiotic and mitotic division: the only distinction is that the chromosome number is half of the number the cell had prior to meiosis. Each chromosome (homolog) is composed of two chromatids, and during Anaphase II, the two chromatids of each chromosome move apart and become separate chromosomes. 2

There is a shift in the terminology applied to these units. While the two chromatids remain attached at the centromere, they are known as chromatids. Immediately upon separating, each chromatid becomes known as a chromosome and is no longer referred to as a chromatid. This is the reason that a cell can divide one chromosome (with two chromatids) into two cells, each with a chromosome - the term applied to the chromatid is changed.

Damage induced during division

In 1949, Levan developed what was to become known as the Allium test for chromosome damage. Growing roots from onion bulbs were soaked in various agents and analyzed for their effect on mitosis. It was discovered that caffeine, for example, caused complete inhibition of mitosis, primarily through the inhibition of cell plate formation.

This test was later used extensively by B.A. Kihlman and extended to other higher plants. Kihlman found that 1 to 24 hour treatments of cells with caffeine and related oxypurines not only inhibited mitosis, but also induced signficant chromosome alterations (aberrations). Specifically, this treatment induced "stickiness" and "pseudochiasmata." Stickiness is the clumping of chromosomes at metaphase and the formation of chromatin bridges at anaphase. Pseudochiasmata is the formation of side-arm bridges during anaphase. Caffeine also causes the formation of other chromosome and chromatid breaks and exchanges.

Colchicine, a drug which inhibits spindle fiber formation during mitosis, can be added to the growing cells to halt cell division at metaphase. This often will result in a doubling of the chromosome number, since colchicine typically inhibits cytokinesis, but not karyokinesis. The doubled chromosomes will fuse within a single nucleus, thus increasing the ploidy value of the nucleus.

Moreover, methylated oxypurines (caffeine, theophylline, 8-ethoxycaffeine) are inhibitors of cell plate formation. Treatment with these agents for 0.5 - 1 hour, with concentrations as low as 0.02-0.04%, results in the cell's failure to undergo cytokinesis; in addition, the nuclei do not fuse into a single unit. Thus, treatment with any of these agents should result in binucleate or multinucleate cells.

Some antibiotics (azaserine, mitomycin C and streptonigrin) have been known to have chromosome-breaking properties, usually associated with G1 or G2 of Interphase. G1 inhibition would result in chromosome breaks, while G2 inhibition would result in chromatid breaks.

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In addition, alkylating agents such as (Di(2- chloroethyl)methylamine or nitrogen mustard, Di(2.3-epoxypropyl) ether (DEPE) and β-Propiolactone (BPL)), Nitrosocompounds (N-Nitroso-N-methylurethan (NMU)), N-Methyl-phenyl-nitrosamine (MPNA), N-hydroxylphenylnitrosamine-ammonium (cupferron) and 1-Methyl-3-nitrosoguanidine (MNNG) have all been indicated as potent chromosome-breaking agents. Other compounds have included such things as maleic hydrazide, potassium cyanide, hydroxylamine, and dyes such as acridine orange in visible light.

The damages involve abnormal metaphases, isochromatid breaks, chromatid exchanges and anaphase bridges to name a few.

Figure 11.5 illustrates some abnormalities induced in plant chromosomes by these compounds.

Exercise 11.1 - Mitosis

LEVEL I

Materials

- Prepared slide of Onion root tip
- Prepared slide of whitefish blastula
- Microscope

Procedure

1. Obtain a slide of an onion root tip and examine it for the basic stages of mitosis. Refer to Figure 11.3 for comparison.
2. Draw and label each of the stages.
3. Obtain a slide of a whitefish blastula for observation of the stages of mitosis in an animal cell. Since early embryogenesis involves rapid cellular division, the whitefish blastula has long served as a model of mitotic division in animals. It also has the advantage of demonstrating clear spindle formation in the cytoplasm.
4. Using Figure 11.3A as a guide, draw and label all stages of mitosis, including spindle formation, in the whitefish blastula.
5. Compare the division of the cell (cytokinesis) from the onion root tip to that of the whitefish blastomeres.

Exercise 11.2 - Grasshopper Spermatogenesis

LEVEL I

Figure 11.6 Stages of meiosis in grasshopper testis

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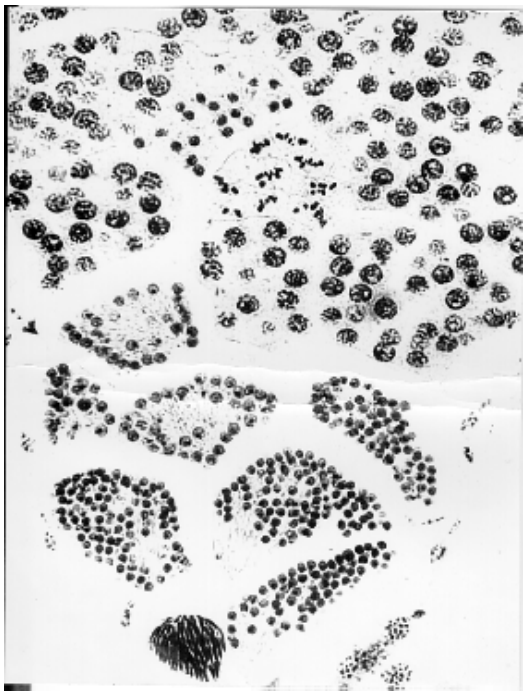

Materials

Prepared longitudinal section of grasshopper testis

Microscope

Procedure

1. Place the slide on the microscope and with low power identify the apical end of the testis and the region where the testis joins with the vas deferens. The apical end is round and packed with cells, while the opposite end is a more open cavity lined with formed sperm.
2. Identify the individual compartments of the testis, known as cysts. The cysts are separated by connective tissue walls or septa.
3. At the apical end, the cells are grouped into presumptive germ cells known as spermatogonia. These cells are undergoing mitosis and are giving rise to all of the remaining germ cells in the testis. Moving from the spermatogonia in the apical end to the vas deferens at the opposite end of the testis, the cells mature as a group. That is, all of the cells within a given cyst will be in the same approximate stage of meiosis. A good longitutinal section will have nearly all of the stages of meiosis displayed on one section, but you may have to examine several slides to observe all of the stages.
4. Using Figure 11.6 as a guide, identify , draw and label each of the following stages:

    Spermatogonia
    Primary spermatocytes in the following phases of Prophase I

        leptotene
        zygotene
        pachytene
        diplotene
        diakinesis.

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Secondary spermatocytes
Spermatids
Sperm

Exercise 11.3 - Ascaris Oogenesis

LEVEL I

Materials

- Prepared slides of *Ascaris megalocephala* maturation
- Microscope

Procedure

1. Place the slide(s) of *Ascaris* on the microscope and focus on the eggs within the body. Unlike the grasshopper testis where division is continuous, the ascaris egg halts its meiotic division until fertilized by a sperm. Subsequently, the final stages of meiosis are completed and nuclear fusion occurs. Within an average cross section of the female, it is usually possible to observe all of the stages of meiosis and early cleavage.
2. Using Figure 11.7 as a guide, identify, draw and label the following:

   Sperm penetration
   First polar body formation (first maturation division)
   Second polar body formation (second maturation division)
   Pronuclear stage (fusion of egg and sperm nuclei)
   First cleavage division (beginning of mitosis)

3. Note the presence of any spindle fibers, and the shape and location of the chromosomes during each phase of development.

Exercise 11.4 - ^{3}H-Thymidine Uptake by Cultured Cells

LEVEL II

Materials

Fibroblast cells in log phase growth

Ca^{++}, Mg^{++} free-phosphate buffered saline (PBSA)

5% (w/v) Glutaraldehyde (GTA)

2% (w/v) Perchloric Acid (PCA)

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Subbed slides (coated with chrom alum gelatin) and Permount

Nuclear Track Emulsion (Kodak or Ilford)

Darkroom and chemicals for photographic processing

- Dektol developer
- Kodak Fixer

Giemsa stain, graded series of alcohols, xylol

Procedure

1. Grow either L cells (Mouse fibroblasts) or chick embryo fibroblasts on coverglasses and then give them ^{3}H-thymidine for a short period of time.
2. At the end of the labeling period, wash the coverslips in PBSA by gently grasping a coverslip with forceps and passing it through a beaker of saline.
3. Fix cultures in glutaraldehyde for 15 minutes.
4. Wash in several changes of water.
5. Wash in cold 2% PCA for 5 minutes to remove unincorporated labeled precursors to DNA. Repeat twice.
6. Wash in water 5 minutes. Repeat.
7. Dry the backs of the coverslips with filter paper, and mount CELL SIDE UP on slides with Permount.
   The slides should be very clean. Coat the slides beforehand with chrom alum gelatin (CAG) by dipping the slides into CAG solution and draining until dry. This coating, and the gelatin coating on the coverslips, help to prevent the emulsion layer (below) from pulling away from the slide during later development.
8. Allow Permount to dry overnight.
9. In a darkroom, spoon out a small amount of gel into a suitable vessel, and slowly melt it at 45° C in a water bath.
   Kodak NTB-3 emulsion is stored refrigerated as a gel in a screw cap bottle inside a double light-tight box.
10. Dip the slide in the emulsion and drain momentarily. Place the slides vertically on a test tube rack in an oven set at 28° C for at least 1 hour in darkness.
    In general, the slides should be dried at a temperature greater than will be used for developing. This minimizes undesirable separation of emulsion from the slide.
11. Place the slides in light-tight boxes containing dessicant and store at 4° C.
12. Develop a sample slide in Dektol diluted 1 part developer to 2 parts distilled water at 18° C for 90 seconds. The temperature of the developer will control the size of the silver grains. Increased length increases background fog of development.

    Develop the slides as indicated in Chapter Two. The length of exposure (refrigerator storage) must be determined for each system (a function of specific activity of label in medium, pool sizes, length of labeling, synthetic rate, etc.). Thus extra control slides are always included to allow repeated sample developing until a useful number of silver grains have accumulated.

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13. Pass the slides through two changes of distilled water, and into photographic fixer at 18° C. Fix for 5 minutes.
14. Wash slides in two changes of distilled water, for a total of 5 minutes.
15. Stain the cells with Giemsa diluted 1:30 as required, or dry slides slowly in a dust-free atmosphere, and stain later.
16. Wash off excess stain briefly in distilled water, dip slide briefly in 70% ethanol, dehydrate in 95% and 100% alcohol, and clear in xylene. Mount coverslip with Permount.
17. Examine the slides with a microscope at 10X magnification and look for clusters of silver grains over the cells. Count and calculate the percent of cells that are labeled.
18. Examine the slides at 40X magnification. Count the number of grains per cell.
19. Prepare a histogram by plotting the number of cells versus the number of silver grains.

Exercise 11.5 - Timing of Cycles

LEVEL III

Materials

- Monolayer cultures grown in 75 mm^2 culture flasks (Cells from Exercise 11.4 may be used, or cultures of tetrahymena, yeast, or algae may be used.)
- ^{3}H-thymidine with at least 4 µc/ml, 0.36 c/mM
- Phosphate buffered saline (PBS), Trypsin
- Methanol:Acetic acid (3:1) fixative
- Nuclear track emulsion and equipment for autoradiographic analysis
- Subbed slides, coverslips, permount
- Giemsa stain
- Microscope (Phase contrast if total cycle is to be measured)
- Clinical centrifuge

Procedure [3]

1. Expose log cultures of cells [4] to ^{3}H-thymidine for a period of 30 minutes. The Mean Cycle Time (hours) of the culture must be known. [5] Calculate the MCT by plotting the growth of the culture and determining the average time for the cell population to double.
2. Pour off the radioactive media (discard with radioactive wastes) and wash cells twice with PBS. Add washings to radioactive waste.
3. Add 1.5 ml of 0.25% trypsin to the flask to dislodge the cells from the flask. Add 10 ml of PBS, mix and pour into a centrifuge tube.
4. Centrifuge in a clinical centrifuge at 600 RPM for 5 minutes to pellet the cells.
5. Aspirate the supernatant, leaving about 0.5 ml of cells packed in the bottom of the tube. Resuspend in PBS to wash, and collect again by centrifugation at 600 RPM for 5 minutes.
6. Aspirate all but 0.5 ml of the PBS from the tube. Gently stir the cells by tapping the centrifuge tube and add 5.0 ml of freshly prepared fixative, drop by drop, gently mixing between each drop. Allow the cells to fix for 1-2 hours.
7. Collect the cells, rinse once with fresh fixative and pellet cells into a final volume of about 0.5 ml of fixative.

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8. Use a pasteur pipette to transfer the cells onto clean, subbed slides and allow to air dry.
9. Prepare slides for autoradiographic analysis as in Exercise 11.4. Coat with nuclear track emulsion and expose for one week. Develop autoradiograms and stain lightly with Giemsa as directed in Exercise 11.4. Prepare permanent slides by attaching coverslips with Permount.
10. Examine the slide and count the total number of cells in interphase and the number of cells that are radioactively labeled. Express this number as a decimal fraction (i.e. if 45% of the cells are labeled, the fraction is 0.45).
11. Use the following formula to compute the length of the S phase.

Time for S phase = Mean Cycle Time x Fraction of Labeled Cells

Optional

The entire process can be repeated with exposure to the radioactive thymidine followed by a brief period of exposure to non-radioactive thymidine. Fix a series of cultures at half hour intervals after removal of the pulsed radioactive label. Expose the cultures to nuclear track emulsion and examine for labeled mitotic images (as opposed to interphase cells). The time between the appearance of the first mitotic cells with label and the level of 50% of the mitotic images labeled represents an approximation of G2.

The length of the mitotic division can be measured directly with phase contrast microscopy (usually less than 1 hour). Estimate G1 by subtracting the time for mitotic division, G2 and S from the Mean Cycle Time.

Exercise 11.6 - *Vicia Faba* and Chemical Damage

Level III

Materials

- Growing root tips of the broad bean,*V. faba* (2n=12)
- Solutions of chromosome-damaging agents listed in the introduction to this Chapter
- Reagents for Feulgen stain (Exercise 2.5)
- Oven at 60° C
- Microscope

Procedure

1. Place freshly germinated bean seedlings into petri plates containing serial diluted samples of suspected chromosome damaging agents.
2. Remove a seedling, rinse with distilled water and cut off root tips. Place the root tips into 1 N HCl, 60° C, for 10 minutes.
3. Rinse the root tip and place in Schiff's reagent in dark for 30 minutes.
4. Rinse the tip again, blot it gently and very gently rub the extreme tip of the root to remove the root cap.
5. Place the root tip into a drop of 45% acetic acid on a clean slide and macerate the tissue with a razor blade.

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6. Place a coverslip over the macerated tissue and squash as in the procedure for the Drosophila polytene chromosomes (Chapter Ten).
7. Identify as many types of chromosome damage as found, referring to Figure 11.5. Draw and label representative views.
8. Use the caffeine treated cells to count the number of abberrations appearing per 100 anaphases examined and record the data in this manner (i.e. number of abnormalities per 100 anaphases examined).

Endnotes

---

1. The phases of Prophase I are named for the appearance of a thread-like structure, known as *-nema*. Leptonema means "thin thread" and leptotene is the adjective applied to the term stage, i.e. proper terminology is the leptotene stage of Prohase I. The word stage is often omitted.
2. <>There is a shift in the terminology applied to these units. While the two chromatids remain attached at the centromere, they are known as chromatids. Immediately upon separating, each chromatid becomes known as a chromosome and is no longer referred to as a chromatid. This is the reason that a cell can divide one chromosome (with two chromatids) into two cells, each with a chromosome - the term applied to the chromatid is changed.
3. From J.H. Priest. *Medical Cytogenetics and Cell Culture*, 2nd ed. Lea & Febiger, Philadelphia, 1977.
4. Cells from Exercise 11.4 may be used, or cultures of tetrahymena, yeast, or algae may be used.
5. This is calculated by plotting the growth of the culture and determining the average time for the cell population to double.

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Chapter 12: Cell Cultures

Introduction

A major advance in our knowledge of cells came about with the ability to maintain them in continous culture. Prokaryotes have been cultured for a relatively long time, but eukaryotic cultures were first accomplished in the early 1900's (Harrison, 1 Carrel 2), with major advances being made only in the past two or three decades

The procedures employed during this exercise will examine several types of prokaryotic cultures as well as a eukaryotic suspension culture, and establish a culture of embryonic fibroblasts. The prokaryotic cultures and the eukaryotic suspension culture are "established" while the embryonic culture will be a "primary" culture. Chick embryos are used for this latter procedure because of the relative ease of culture, ease of obtaining embryos, and relative simplicity of their nutritional needs. Highly differentiated cells would be more difficult to establish and in some cases not possible at all (given current technology).

Prokaryotes

Prokaryotes are cells without nuclei and are generally considered to be more primitive than eukaryotes (cells with true nuclei). Typically, prokaryotes are easier to culture in a laboratory because most of them have less stringent nutrient requirements. For this lab we will utilize bacterial cultures grown in nutrient agar, an environment in which human pathogenic organisms are extremely unlikely to grow.

Bacteria may be examined by observing the living, unstained microbes in a wet mount (phase contrast or dark field illumination), by observing dead cells stained with dyes under bright field illumination, or by observing cells prepared for electron microscopy. Our procedures will use "fixed" (i.e. dead) cells stained for standard light microscopy.

Figure 12.1 Bacterial growth curve

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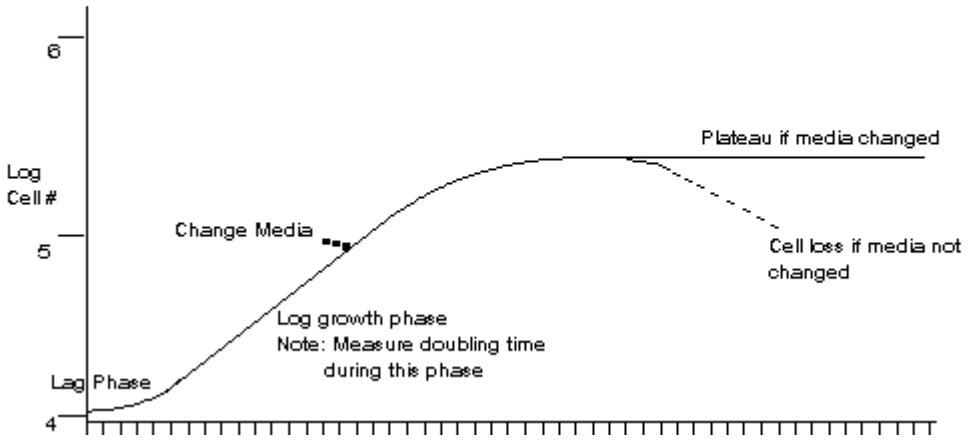

When cells are grown, they will have specific growth characteristics, depending on the media, the temperature and the strain of cells utilized. For bacteria (and some algae, fungi and other eukaryotic tissue culture) it is possible to measure the growth of cell populations by calculating cell number or mass. With modern equipment and the proper computer software, this can be a completely automated analysis, that would include the specific morphological data as well (size, shape and density of colonies or individual cells).

Since bacteria are small, they are difficult to count through direct visualization, but can be counted if one makes an assumption that each bacterium is capable of forming an individual colony. The mass of a bacterial suspension can be deduced from the optical turbidity of a suspension.

Eukaryotes

By contrast to the simple broth cultures of *E. coli*, the nutrient requirements of even the simplest eukaryotic culture are complex. Refer to Table 12.1 for a comparison of the ingredients of Nutrient Broth and Minimum Essential Media (MEM), a typical eukaryotic media. Eukaryotes also require supplemental sources of materials, most often in the form of blood serum. Fetal calf serum is used extensively for this purpose, since it is readily available, and the fetal nature of the serum limits the presence of antibodies, which might negatively effect cell growth.

Our first procedure will involve the simple transfer of an established culture from a suspension culture. An aliquot will be removed from a commercially available cell line and transferred to prepared transfer vessels. Each day students will observe these transfer cultures with an inverted phase contrast microscope, and remove aliquots for cell counting with a hemacytometer. Simultaneously, they will check on the viability of the cells through a dye exclusion technique.

The second procedure will be somewhat more complicated. Students will remove chicken embryos from the egg, trypsinize them to disaggregate the cells, and transfer the resulting cells to culture flasks. This procedure establishes a primary culture, or one that is a first generation growth from "in vivo" cells. Established culture lines are the result of long term selection for cells capable

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of growth under "in vitro" conditions. As such, they are more consistent clones, but often have genetic and structural alterations that differ significantly from the starting cell lines.

Table 12.1 Eukaryote and prokaryote culture media

As the cells grow in culture, we can observe three distinct phases. The first is a Lag Phase, usually no more than 1-2 days in length, and during which there is little or no increase in cell number. During this time, the cells are "conditioning" the media, undergoing internal cytoskeletal and enzyme changes and adjusting to the new media.

This is followed by a Log Phase. During this phase the cell number increases exponentially. This growth will continue as long as there is sufficient nutrient to support the increasing cell number. Eventually some critical nutrient will become limiting, however.

The final phase is the Plateau Phase. During this phase the number of cells remains constant (although not necessarily viable). Eventually, of course, the cells will die unless subcultured or fresh media is added. The final procedure in this laboratory exercise involves establishment of a primary culture from a chick embryo. Here, the cells are not established in culture, but must be disaggregated (detached from each other) and placed into a foreign environment (the culture media). Disaggregation in embryos is reasonably easy, but depends on the enzymatic dissolution of the cells glycocalyx, and the disruption of many plasma membrane structures and chemical elements. Consequently, the cells will take a longer time before they grow (that is, there is a long Lag Phase), and the selection process will favor cells which grow in contact with the culture vessel surface.

The cells will continue to grow in contact with the vessel and give rise to a "monolayer" culture. The cells will cease to divide when they reach confluency; they are said to demonstrate contact inhibition.

Consequently, growth curves are not measured by removing aliquots and determining cell concentration, but are measured by the density of cells growing on the vessel surface. This is accomplished through the use of an ocular grid inserted into an inverted phase contrast microscope. Cell density (cells/cm^2) is then plotted on a log scale against time in culture.

Figure 12.2 Eukaryotic growth curve

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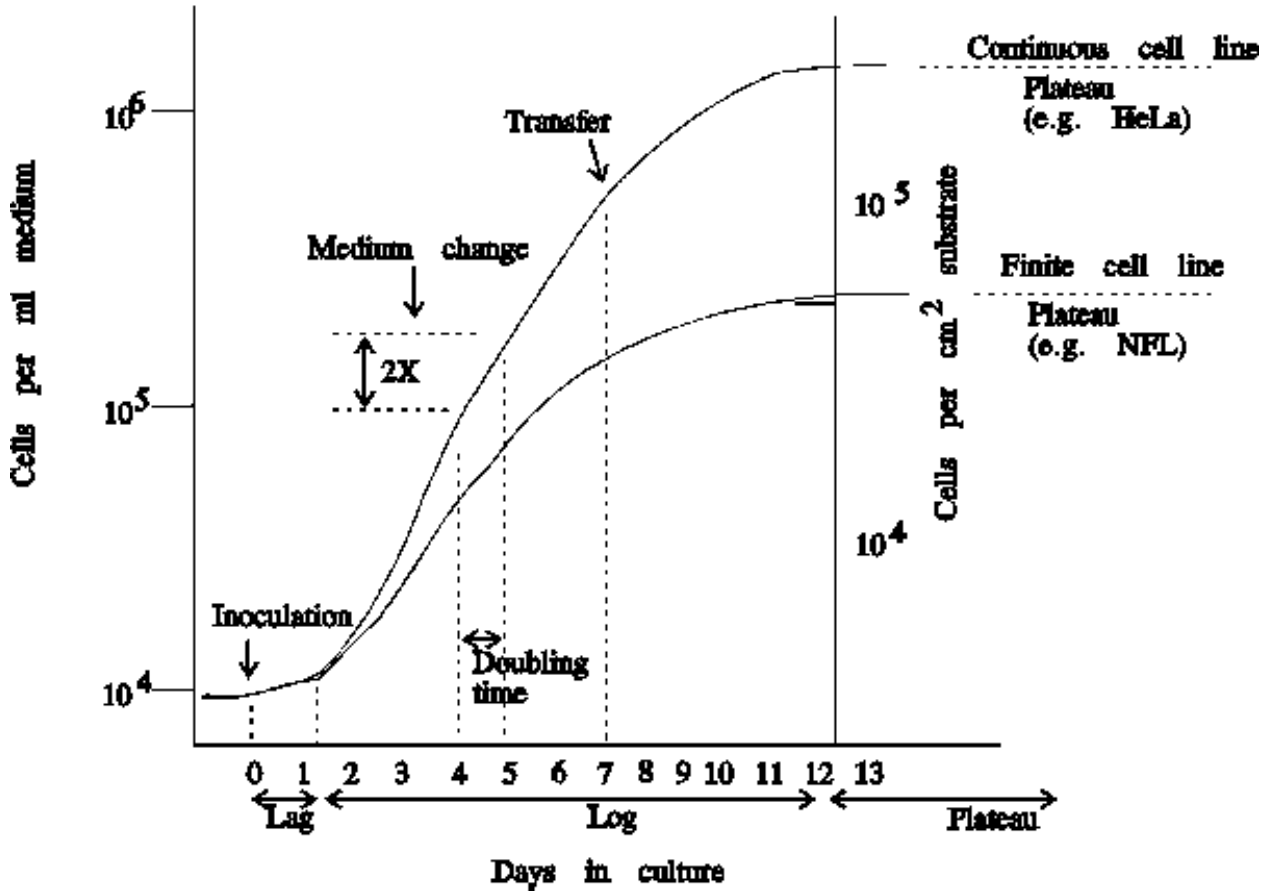

Exercise 12.1 - Aseptic Cell Transfers

Level I

Materials

- Bunsen burner
- Wire loop
- Petri plate or broth tube
- Bacterial culture
- Microscope slides

Procedure

1. Pick up the inoculating loop and hold it pointed down into an open flame until the loop glows red. This process sterilizes the loop of wire and is known as "flaming" the loop. It will result in a sterile loop and will not contaminate your stock culture. If there are liquids already on the loop, the loop should be gradually placed into the flame to dry the loop. If the loop is rushed into the flame, the drop of liquid will splatter and spread bacteria over your work surface.
2. Pick up a broth culture in one hand, while holding the loop in the other. With the last two fingers of the hand holding the loop remove the cap from the culture and gently flame the top of the test tube (do not overheat). Insert the flamed inoculating loop into the test tube until it is submersed in the broth. The

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loop should be allowed to cool slightly before immersion. Retract a small quantity of the broth held in the loop and replace the cap on the culture.

3. Open the top of your transfer vessel (tube with water or nutrient agar) and flame the open top of the tube. Insert the loop into the tube and into the liquid in the tube. Withdraw the inoculating loop slightly from the liquid, blot gently on the inside of the tube and completely remove from the tube. Replace the top on the tranfer tube.
4. Immediately flame the inoculating loop.

Exersise 12.2 - Examination of Bacterial Colonies

LEVEL I

Materials

- Petri plate cultures of various bacteria

Procedure

1. Obtain an established culture. Note that the bacteria grow in clearly defined groups, known as colonies. In most cases, each colony is the outgrowth from an individual cell, although they may overlap if excessive numbers of cells were plated.
2. Visually examine the individual colonies of bacteria and describe them according to the following characteristics:
    - Size. Pinpoint, small, medium, or large, based on the relative differences between the largest and smallest colonies seen.
    - Shape and Margins. Round, regular or irregular.
    - Elevation. Flat, convex or rounded, umbonate (flat on margins and raised in the center - like a fried egg), craterlike (with depressed center).
    - Consistency. Shiny or rough.
    - Color. Describe the color as accurately as possible, distinguishing between different types of gray or white, yellows, and red. If the pigment appears to diffuse into the surrounding medium, rather than coloring only the colony, it is a water-soluble pigment.
3. Determine and record the identity of your colonies.

	Colony 1	**Colony 2**	**Colony 3**	**Colony 4**
Size				
Shape				
Margins				
Consistency				
Color				

Exercise 12.3 - Gram Stain (+\-)

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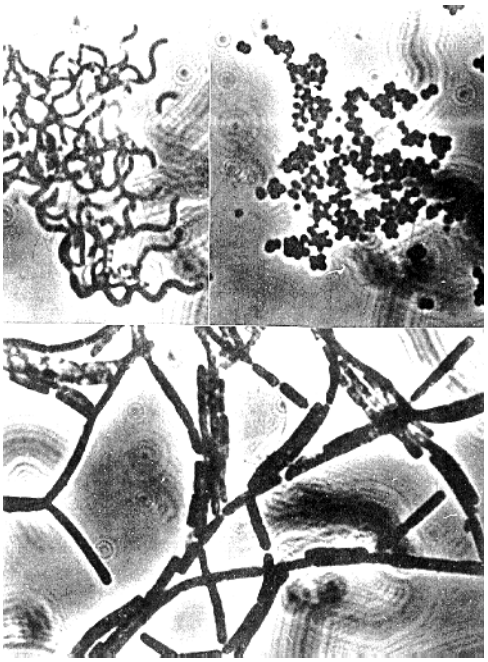
LEVEL I

Figure 12.4 Typical bacterial shapes



Materials

- Colonies of bacteria from Exercise 12.2
- Toothpicks
- Crystal violet
- Gram's iodine
- 95% ethanol
- Safranin
- Microscopes with oil immersion

Procedure

1. Before staining the individual colonies, you should first practice the technique by observation of the gram positive micro- organisms normally found in the gum linings of your mouth.
2. Use a clean toothpick to rub along the gingival crevices (area between tooth surface and gums) of your mouth.

   Rub lightly!

3. Mix the scrapings with a drop of water previously placed on a clean slide, spread in a thin film over the center of the slide and allow to air dry.
4. Fix the smear to the slide by passing the slide (smear side up) quickly through a flame three times. If the slide is held directly in the flame, it will heat up too rapidly and break. The trick is to gently dry the smear without overheating the slide.
5. Place the slide on a staining rack. Apply the stains on the fixed smear as follows:

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- Flood the slide with crystal violet for 30 sec.
- Rinse with water.
- Flood with Gram's iodine for 60 sec.
- Rinse with water.
- Decolorize with 95% ethanol.
- Rinse with water.
- Counterstain with safranin for 60 sec.
- Rinse with water and blot dry (no rubbing!).
- Examine under oil-immersion objective lens. 3

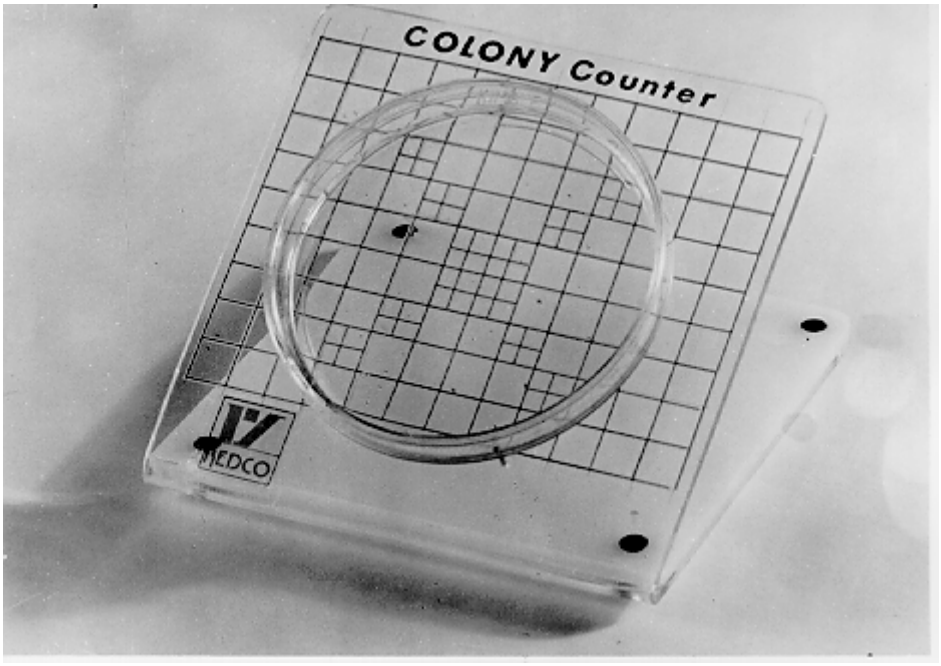
Gram-positive bacteria retain crystal violet after washing with 95% ethanol, while gram-negative bacteria lose the purple dye after washing with 95% ethanol. The positive or negative reaction is a measure of the presence or absence of specific polysaccharide components of their cell walls. Safranin is used as a pink counterstain, so that Gram - cells can be visualized. In practice then, the distinction is made between purple cells (+) and pink cells(-).

6. Determine the basic cell shape of the bacteria.
7. Add the information on Gram stain and cell shape to the work done in Exercise 12.2.

Exercise 12.4 - Prokaryote Cell Number by Dilution Plating

LEVEL II

Figure 12.5 Colony counter



Materials

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- Broth culture of *E. coli*
- Tubes of nutrient broth (9.9 ml each)
- Nutrient agar plates
- Sterile transfer pipettes (1.0 ml)
- Quebec colony counter (Optional)

Procedure

1. Obtain a broth culture of *E. coli* and carefully mix the contents to ensure equal suspension of the bacteria.
2. Obtain four test tubes each containing 9.9 ml. of nutrient broth. These will be used to produce a serial dilution of the stock culture.
3. Using sterile pipettes, remove 0.1 ml of well suspended cells from the stock culture and transfer these aseptically to one of the waiting test tubes of broth. This tube now contains a dilution of 1/100 or 10^{-2}. Gently but thoroughtly mix the contents and label this tube at 10^{-2}.
4. Repeat this process, but now aseptically remove 0.1 ml of culture from the 10^{-2} tube and place it into a new broth tube, which now becomes a 1/10,000 or 10^{-4} dilution. Mix the contents and label as 10^{-4}.
5. Repeat this procedure twice more to produce respectively a 10^{-6} and a 10^{-8} dilution. Be sure to mix thoroughly and label each tube.
6. You should now have a serial dilution of the stock culture with tubes at 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8} . The original stock culture will almost invariably be too high a population for the next step, so you will only use the four dilutions that you have produced.

   Using four separate petri plates containing 15 ml. of nutrient agar each and four separate sterile pipettes, transfer 1.0 ml of each dilution broth suspension onto the surface of a petri plate. Carefully label the plates and place them in an incubator for 24 hours at 37° C.

7. At the conclusion of incubation, remove each of the four petri plates and count the number of colonies formed on the plates. For proper statistical analysis, the plate containing between 30 and 300 colonies will give the most accurate results.

   The colonies can be more easily counted by using a Quebec Colony Counter which allows proper illumination, a grid overlay and by slight magnfication of the plate surface.

8. Multiply the number of colonies counted by the dilution factor to obtain the population density of the original broth culture.

Notes

A growth curve can be established by repeating this procedure every two hours. Since the number of bacteria can be large, it will be necessary to plate cultures serially diluted. Count the number of colonies for each dilution and average the results.

Exercise 12.5 - Cell Mass by Measurement of Turbidity

LEVEL II

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Materials

- Trypticase soy broth culture of *E. coli*
- Tubes of trypticase soy broth (9.9 ml each)
- Tubes of trypticase soy broth (5.0 ml each)
- Nutrient agar plates
- Sterile transfer pipettes
- Spectrophotometer and cuvettes 4

Procedure

1. Obtain a trypticase soy broth culture of *E. coli*. Trypticase broth is better than nutrient broth, since it is inherently less light absorbent.
2. Prepare trypticase soy broth dilutions of 10^{-3} and 10^{-6} of an appropriate bacteria culture, as described in Exercise 12.4.
3. Now, for each of the two dilutions, set up a second series of dilutions, this time diluting by 1/2 each time. That is, transfer 5.0 ml of the 10^{-3} culture to 5.0 ml of fresh broth and mix thoroughly. Use 5.0 ml of this and transfer to 5.0 ml of fresh broth to produce a 1/4 dilution. Repeat for 1/8, 1/16, and 1/32 dilutions. Repeat the entire 1/2 dilution series for the 10^{-6} dilution.

   You should now have twelve tubes, six for each of the 10^{-3} and the 10^{-6} dilutions. These are then diluted 1/2, 1/4, 1/8, 1/16, and 1/32.

4. Use 1.0 ml of each of the twelve dilutions and plate on nutrient agar plates to perform a colony count as in the preceeding section.
5. Set a spectrophotometer for 686 nm wavelength and be sure it is turned on and functioning properly. Adjust the dark current to 0% T.
6. Place a cuvette containing trypticase soy broth into the spectrophotometer and adjust the reading to 100% T. This is the blank for all subsequent measurements.
7. Transfer the remaining contents of the 12 dilutions to spectrophotometer cuvettes and measure the %T for each. Compute the absorbance for each sample. 5
8. Count the number of colonies formed for each sample after 24 hours. of incubation.
9. Plot the absorbance of each sample against the plate count for that sample.

   Once this has been accomplished, you will have a value for computing the cell population number directly by measuring the absorbance of a suspension. This can be more readily measured on a continuous basis. To do this, grow suspensions directly in cuvette tubes and measure the A_{686} at timed intervals. Record a growth curve. By connecting a recorder to a spectrophotometer and keeping the chamber at 37° C, a continuous growth curve can be automatically recorded (assuming care is taken to ensure proper suspension of the bacteria throughout the time period involved). Lacking such sophisticated equipment, the tube can be removed from an incubator at intervals, gently suspended, measured for absorbance and returned immediately to the incubator. Plot cell growth against time to produce a growth curve.

Exercise 12.6 - Transfer of Eukaryote Suspension Cultures

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LEVEL II

Materials

Fibroblast suspension culture

Tissue culture laminar flow hood

Media appropriate to culture line used

Disposable pipettes (10 ml and 1.0 ml)

Disposable culture flasks

Procedure

1. Obtain a culture of mouse fibroblast cells in suspension culture. This will be a simple culture with minimal requirements, and one selected for excellent growth characteristics. The transfer procedure will be similar to that for prokaryotes, with a few major changes. First, all transfers will be done in a tissue culture hood in order to maximize asepsis. Secondly, the cells will be transferred with siliconized pipettes rather than wire loops. The silicone prevents adherance of the cells to the glass wall of the pipette.

   A tissue culture hood is a device that has air moving in layers and under positive pressure. Since the air is filtered, it contains minimal numbers of bacteria or fungal spores, and since it is under a positive pressure, those particles that are present are blown out of the hood. The layering prevents airborne organisms from settling on the work surfaces.

2. Arrange the materials in front of you, easily accessible through the opening of the tissue culture hood. Ensure that any alcohols and wrapping paper are kept clear of the bunsen burner. Pre-sterilize the hood before use, and use disposable sterile gloves.
3. Loosen the cap of a tissue culture flask and the cap of a stock bottle of tissue culture media. 6

   Insert the tip of a sterile pipette into the stock bottle and remove 10 ml of media. Transfer the media to the tissue culture flask.

4. Open the top of the suspension culture and use a sterile 1.0 ml transfer pipette to remove a 1.0 ml sample of the culture. Transfer it to the fresh media in the culture flask. Secure all caps that have been loosened.
5. Place the new cultures in an incubator at 37° C.

Exercise 12.7 - Viability Cell Count

Materials

- Suspension culture of cells

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- Sterile transfer pipettes
- Stock 0.2% (w/v) Trypan blue
- Hemacytometer and microscope

Procedure

1. Gently swirl a suspension culture to distribute the cells evenly. Aseptically remove a small sample (0.1 ml) of cells from the cultures. Place the sample in a separate test tube (it need not be sterile).
2. Dilute 4 parts of stock Trypan Blue with 1 part of 5X saline and add 0.1 ml of the diluted dye to your sample. Mix gently.
3. Set up a hemocytometer and cover slip. Immediately place a drop of the stain/culture combination on the hemocytometer (remember to use both sides of the hemocytometer) and wait one minute.
4. Observe the cells with low power microscopy. Count the total number of cells, and the number of stained cells.
5. Compute the concentration of viable cells per ml. of culture.

Notes

Trypan Blue is a stain that is actively extruded from viable cells, but which readily enters and stains dead cells. Therefore, the cells which are blue are dead. The difference between the total number of cells and the number of dead cells would be the number of viable cells in a given aliquot of your culture. Trypan Blue actually significantly overestimates the number of viable cells, but is sufficient for purposes of this lab.

Approximately 30% of the cells measured as viable with Trypan Blue will not be able to continue growth beyond a 24 hour period.

Exercise 12.8 - Computation of Transfer Aliquots

LEVEL II

Materials

- Suspension culture of cells
- Media appropriate to culture
- Culture flasks
- Transfer pipettes
- Hemacytometer and microscope

Procedure

1. Obtain 3 transfer culture vessels. The vessels may already contain fresh culture media, or you may be asked to transfer your own.
2. Obtain a suspension culture and count the number of cells/ml of culture using the procedure listed in Exercise 12.7.
3. Compute the volumes of the suspension culture needed so that when added to 25 ml of fresh media, the

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final concentrations will be:

1 X 10^5 cells/ml
5 X 10^4 cells/ml
1 X 10^4 cells/ml

For example: If you have 25 ml of fresh media, you will need to add 25 X 10^5 cells to obtain a final concentration of 1 X 10^5 cells/ml If your suspension culture contains 5 X 10^6 cells/ml, you will need to transfer 5 X 10^{-1} ml or 0.5 ml of culture to the fresh media (25 x 10^5 cells divided by 5 x 10^6 cells/ml).

Use the formula:

Aliquot to be transferred = Number of cells to be transferred /Culture concentration

4. Seed three flasks each containing 25 ml of fresh media to a final concentration of 10^5 cells/ml, 5 X 10^4 cells/ml and 10^4 cells/ml.
5. Label and place your fresh cultures in the tissue culture incubator at 37° C.

Lay the culture flasks on their sides in order to maximize the air exchange surface of the culture and to prevent the cells from drowning. An alternative would be to incorporate mechanical shaking of some type.

Exercise 12.9 - Eukaryote Growth Dynamics

LEVEL II

Materials

Suspension cultures set up from Exercise 12.8

Sterile transfer pipettes

Materials for viability counting (Exercise 12.7)

Procedure

1. After 12 hours, aseptically remove 0.1 ml from each of the three cultures, add 0.1 ml of trypan blue and count the total number of cells and the number of blue cells. Compute the number of viable cells/ml.
2. After 24 hours (from the time of seeding), repeat step 1.
3. Continue to repeat step 1 at 24 hour periods (i.e. daily) until there is no change in the number of cells/ml of culture.
4. Plot cell concentration on a log scale vs time of culture. Identify and label the Lag, Log and Plateau phases for your culture.
5. Select a period of time during the Log Phase and compute the doubling time for your culture. That is, the time required during the Log Phase to exactly double the number of cells/ml.

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Exercise 12.10 - Establishment of a Primary Culture

LEVEL III

Materials

- Chick embryo (approximately 8 days old)
- 70% (v/v) ethanol for swabbing
- Sterile scissors, forceps and probes
- Sterile petri plates
- Phosphate buffered saline (PBS)
- Trypsin, cold sterilized in a 125 ml sterile erlenmeyer containing a magnetic stirring bar
- Minimum Essential Medium
- Fetal Calf Serum
- Clinical centrifuge with sterile capped centrifuge tubes
- Culture flasks
- Inverted phase contrast microscope (Optional)

Procedure [8]

1. Candle an 8 day old egg to ensure that it is alive. This is easily accomplished by holding the egg in front of a bright light source; the embryo can be seen as a shadow. Circle the embryo with a pencil.
2. Place the egg in a beaker with the blunt end up, and wash the top with a mild detergent, followed by swabbing with ethanol.
3. Carefully puncture the top of the egg with the point of a pair of sterile scissors and cut away a circle of shell, thus exposing the underlying membrane (the chorioallantois).
4. With a second pair of sterile scissors, carefully cut away and remove the chorioallantoic membrane, exposing the embryo.
5. Identify and carefully remove the embryo by the neck, using a sterile metal hook or a bent glass rod, and place the embryo in a 100mm petri dish containing phosphate buffered saline (PBS). Wash several times with PBS by transferring the embryo to fresh petri plates. After removal of all yolk and/or blood, move the embryo to a clean dish with PBS.
6. Using two sterile forceps, remove the head, limbs, and viscera. Be sure to remove the entire limb by pulling at the proximal end. Move the remaining tissues of the embryo to yet another dish and wash with PBS.
7. Mince the embryo finely with scissors and transfer the minced tissue to a flask containing PBS. Allow the tissue pieces to settle.
8. Remove the PBS with a sterile pipette and add 25 ml of trypsin, a proteolytic enzyme. Stir the solution gently at 37° C for 15-20 minutes.
9. Allow the larger, undigested tissue pieces to settle and decant the supernatant into an equal volume of Minimal Essential Medium (MEM) + 10% Fetal Calf Serum (FCS). FCS contains protease inhibitors which will inactivate the trypsin.
10. Centrifuge the cells in MEM at 1000 rpm for 10 minutes in a standard clinical centrifuge. Remove the supernatant and resuspend the pellet in 25 ml of fresh MEM + 10% FCS.
11. Remove 0.1 ml of the culture and determine cell concentration and viability as directed in the previous

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section.

12. Seed two 25 cm^2 plastic culture flasks containing 25 ml of MEM + 10% FCS to a final concentration of 10^5 cells/ml.
13. Label and place your cultures in the tissue culture incubator at 37° C and examine daily for cell density and morphology.
14. Note any changes in the color of the media. Tissue Culture media has a pH indicator (Phenol Red) added in order to check on the growth of cells. The media initially is a cherry red (with slight blue haze) and turns orange and then yellow as the cells grow, thereby reducing the media. Should this color change occur within 24 hours, the culture is most likely contaminated and should be disposed of.
15. Examine the cultures using an inverted phase contrast microscope. This will allow observation of the cells without opening or disturbing the growth.
16. Make cell density determinations at 10 X magnification using a square ocular grid, as explained in Chapter One for the determination of area.
17. Plot the cell density on a log scale vs. time of culture.
18. Diagram the shape of the cells at each phase.

Notes

The cultures will develop differently than the suspension cultures. The viable cells will grow out of the trypsinized pieces of tissue and will remain in contact with the bottom of the culture flask. They will continue to divide and migrate until the entire bottom of the flask is covered with a single layer of cells (contact inhibition and the formation of a monolayer).

Endnotes

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1. Harrison, R.G. (1907) Observations on the living developing nerve fiber. Proc. Soc. Exp. Biol. Med. 4:140-143.
2. Carrel, A. (1912) On the permanent life of tissues outside the organism. J. Exp. Med. 15:516-528.
3. One of the continuing differences between microbiologists and microscopists is the lack of a coverslip when viewing bacteria. Convenience causes microbiologists to skip the process of placing mounting media and a coverslip on their slides. This causes microscopists to cringe at the thought!
4. If the cultures are to remain aseptic, the cuvettes can be sterilized and plugged. Alternatively, culture flasks with cuvette side arms can be used.
5. Absorbance may be read directly if the spectrophotometer is equipped with digital display. Absorbance is more difficult to interpret on an analog display.
6. It is assumed that the media has been pre-mixed, with serum and other additives put into the media. The media should be in small aseptic containers for student use.
7. From: Barbara B. Mischell and Stanley M. Shiigi. "Selected Methods in Cellular Immunity". W.H.Freeman & Co. San Francisco, 1980, p. 17.
8. Modified from Freshney, R. Ian. *Culture of Animal Cells: A Manual of Basic Technique*. Alan R. Liss, Inc. New York, 1983.

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Chapter 13: Differentiation

Introduction

Differentiation occurs when cells change in structure and function within a period of time. As such, one of the major problems in research is the factor of time. Cells must be "competent" or primed for a differentiating change by being properly "induced" by some internal influence (gene, ion flux, metabolic alteration) or external factor (hormone, growth factor, cell-cell communication). Finally, they must be in the correct association with neighboring cells (position effect in embryos, contact inhibition or guidance) and strata for the change to occur. All of these factors combine to make an afternoon laboratory session on differentiation difficult to coordinate.

Consequently, the laboratory will use prepared slides of materials that have undergone differentiation, so that the time can be compacted into convenient laboratory periods. We will use some living cells by carefully choosing systems which can be closely monitored (slime mold development) or which are slower to develop and thus able to be "ready" at the time of a lab session (fern gametophytes).

We will observe specific molecular alterations indicating differential gene activity through indirect observation of the changes, primarily through the use of inhibitors of the basic DNA/RNA/Protein system.

Embryogenesis

A classical approach to the basic process of cellular differentiation has involved the primary formation of the three germ tissues of embryos, namely ectoderm, endoderm and mesoderm. The sea urchin provides readily available egg and sperm which can be fertilized in laboratory with ease. Further , there is little yolk within the egg, and the yolk is evenly distributed (i.e. an isolecithal egg). The embryo demonstrates "regulative" development (each embryo cell or blastomere has the ability to form a complete organism), it has several mutant strains available for genetic analysis, and it develops in a synthetic sea water environment, without the need for elaborate culture or "in utero" studies. The eggs are large enough to be studied with standard light microscopes and can be conveniently micro- manipulated for nuclear transplant and cytoplasmic injections.

The sea urchin embryo is thus a convenient model system for the complete analysis of early embryogenesis. For our purposes, we will limit the laboratory study to descriptions of the basic processes of cleavage, induction, migration and invagination of cells.

Caenorhabditis elegans is rapidly becoming a favorite organism for early embryogenesis. This invertebrate worm (nonparasitic nematode) reproduces as a self-fertilizing hermaphrodite. The genetics of the nematode is fairly simple; each contains a pair of sex chromosomes (XX = Female, XO = Male) and five pairs of autosomes. The male karyotype develops spontaneously in 1/700

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developing embryos. Males can be mated to hermaphrodites, but hermaphrodites never mate with each other. Figure 13.1 presents the life cycle of C. elegans.

C. elegans has an asymmetric first cleavage which clearly establishes two distinctly different cells, which in turn are the progenitors of specific parts of the final organism. Starting from the single cell zygote, the first stage larva hatches in about 14 hours after fertilization with a total of 546 somatic nuclei and four primordial gonadal nuclei. The embryo is a classic "mosaic" where each cell carries the information for a piece of the whole. Blocking cell division as early as the two- cell stage will result in cells which are unable to form complete organisms, but which will demonstrate specific fates. The cells are each given labels indicative of their ultimate fate, and limited potential.1, 2 Figure 13.2 presents the general scheme for this cellular development.

The eggs of *C. elegans* are small, transparent, capable of developing outside of the mother, have relatively small genomes and have had each cell's development characterized. 3 With the isolation of mutants, and characterization of the specific gene loci (DNA sequencing), powerful probes of early gene control over embryogenesis have become available. 4

The sequence of events in the development of this nematode are observed by the formation of polar bodies and completion of meiosis following fertilization. At this time, we can see intensive cytoplasmic streaming which results in the observable segration of germ line specific granules to the posterior of the embryo. Subsequently, the pronuclei fuse and the first cleavage is initiated, giving rise to a large AB blastomere and a small P-1 blastomere. Antibodies can be made to the P granules and we can observe a clear mosaic pattern distribution can be observed with immunofluoresence.

Hematopoietic System

The development of the blood and related cells has long been a subject of intensive study. One reason is that blood represents a type of differentiation which is continuous throughout the life of an organism, and demonstrates the role of stem cells in development. Stem cells are embryonic derivatives which retain the ability to form clones, yet remain relatively undifferentiated themselves. This is accomplished by the division of a stem cell into two cells, typically one of which will continue on to a highly differentiated role, while the other remains as a stem cell. It is possible for stem cells to divide into two stem cells, and on occasion, a stem cell will divide and form two differentiated cells (thus ceasing to be a stem cell). The hematopoietic system (blood forming) demonstrates each of these modes.

In adults, blood cells do not divide within the bloodstream, but are produced either within the bone marrow, lymph nodes, spleen or thymus. Thus, these organs make up the hematopoietic system, or blood forming system. Once the cells are formed through cellular division, they must mature before attaining their final differentiated state. Smears of bone marrow (and sometimes whole blood) will display many intermediate states of differentiation.

It is generally believed that all blood cells arise from a single type of cell (the "unitarian" theory), which is itself derived directly from embryonic mesenchyme. The cell is known as a hemocytoblast and is characterized by its large size (8-30 microns), minimal quantity of basophilic cytoplasm, lack of cytoplasmic granules, large nucleus and presence of 2-3 prominent nucleoli.

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They are fairly scarce within bone marrow (% of cells present), yet they are the progenitors of all other blood cells.

A hemocytoblast within bond marrow will give rise to granulocytes, megakaryocytes and erythrocytes. The same cell found in lymphoid tissues will give rise to lymphocytes. For the exercises used in this manual, we will limit our observations to those cells found in the bond marrow. The lymphocytic series is more difficult to study since it has fewer distinguishing marks (granules, basophilia). The erythrocyte, granulocyte and megakaryocyte series are clearly defined by observable light microscope characteristics. Collectively they represent a model series of cellular alterations leading toward differentiation from a stem cell population.

Photomorphogenesis of Ferns

Axenic growth of fern prothallia germinated from spores represents a ready sequence of cellular differentiation which has many of the standard characteristics. 5, 6, 7 In addition, the developing gametophyte is haploid (simplifying the genetics), and since the fern fronds grow asexually, usually all the spores from a given field are from the same plant, thus ensuring genetic homogeneity.

As the spore germinates and begins to develop a heart-shaped gametophyte, it will pass through several stages of recognizable development. The first is the formation and extension of a rhizoid, followed almost immediately by cellular division within a single plane. This division takes place within the "tip" cell and gives rise to a filamentous protonema. The tip cell will then alter the planes of division by 90° and subsequently, the growth will become two-dimensional. As this process continues, the typical shape of a young fern prothallus is established. The division continues forming a structure with a single layer of cells, with differentiated areas forming rhizoids, the body of the prothallus, antheridia (sperm) and archegonia (egg). Thus, there are a small number of easily recognizable differentiated states, within an organism that can be propagated from spores with genetic homogeneity and which can be grown easily under aseptic and axenic conditions.

The body of the fern gametophyte can be sliced into various components and a new prothallus will develop from each. The spores can be irradiated and will give rise to tumors, abnormal three-dimensional growths with complex structure and physiology. The cultures of developing gametophytes can, of course, be treated with any number of drugs for molecular analysis of function.

In addition, the developing gametophyte demonstrates a characteristic process of photomorphogenesis. This process is one in which light has an effect upon the structure and function of the plant, independent of photosynthesis. There are several examples of this process, most of which involve red light and far red light wavelengths and the germination and development of seeds. These systems involve the pigment phytochrome, and require somewhat difficult illumination control. The fern gametophyte, by comparison, alters its growth when grown in various components of visible light. Specifically, the shape of the gametophyte will differ when grown in red, blue and green light.

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Under sufficient illumination with blue light, the gametophyte will develop as indicated above, that is, as though it were in white light. Under red illumination (corrected for the same energy intensity), the gametophytes will develop as filaments only. That is, the alteration in the plane of development which gives rise to the two-dimensional growth will not occur. Under green illumination, the gametophyte will grow filamentous, but will also develop and differentiate massive amounts of antheridia. Refer to Figure 13.4 for details of fern gametophyte development.

Cell Communication - *Dictyostelium* and cAMP

The development of the slime mold *Dictyostelium discoideum* has been chronicled for several decades, with the primary establishment of this important system accomplished by J.T. Bonner 8, 9, 10 and followed by too many investigators to list here. One would be remiss, however, to not acknowledge the extensive work of the Sussmans. 11 There has been a more recent review of the growth characteristics in Ashworth. 12

The basic developmental pattern of *D. discoideum* is given in Figure 13.5.

This organism is a cellular slime mold that lives most of its life cycle as a free amoeboid cell. The amoeba of *D. discoideum* function as simple protists, yet when nutrition becomes limited, they can aggregate and form a multicellular slug, with many of the characteristics of a true multicellular organism.

Within its life cycle, the amoeboid cell emerges from a spore, is strictly parasitic on bacteria and divides by simple binary fission. When the number of amoeboid cells increases, and there is a slight drying of the environment, the amoeba will gather, form protective slime sheaths around themselves and begin differentiating. The cells arrange themselves into positions, and the location a cell has will in turn determine its ultimate fate, whether it will become a part of the stalk (prestalk) or will be involved in the sexual reproduction of the species (prespore).

Figure 13.5 Development of *D. discoideum*

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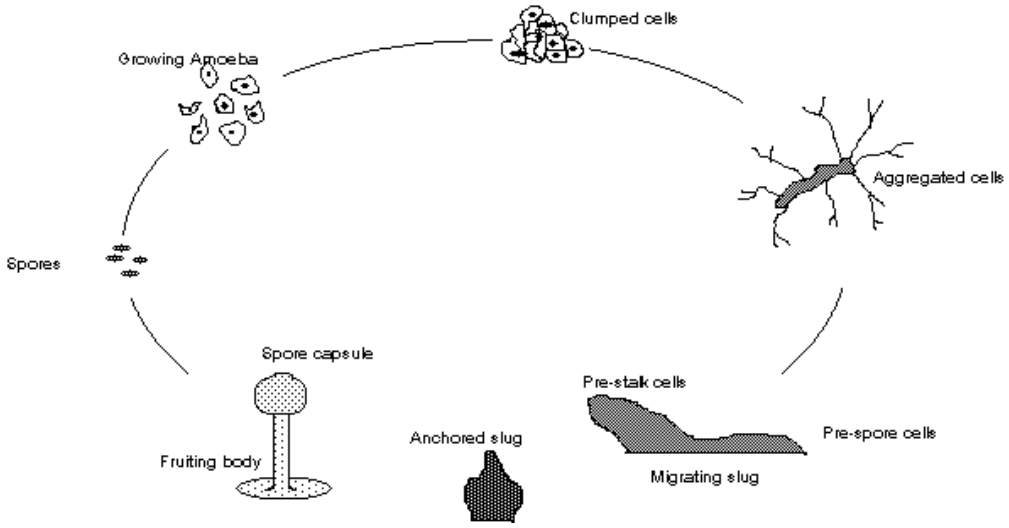

The aggregated amoeba first form a slug or pseudoplasmodium which travels about as though it were a multicellular organism. Eventually, it will settle down on the agar plate (or a leaf if in a lake) and form a base, stalk, and fruiting body (sorocarp). Spores are formed within the fruiting body and the process begins again.

It has long been known that the primary induction of this phenomenon is a pulsed level of the nucleotide cAMP. 13 The level of cAMP that a cell is exposed to is fundamental to its differentiation into either stalk cell or spore cell. 14 Cells can be isolated, which are thus prestalk or prespore, after cAMP is administered.

If agar cultures of the amoeba are supplemented with 10^{-3} M cAMP, and the amoeba are placed in small drops onto the surface of the media (3-8 mm diameter), the cells will form a ring around the drop as they migrate from the center. 15 Within 24 hours, some of the advancing cells within the ring will differentiate into separate stalk cells. By contrast, in the absence of cAMP, the cells aggregate and form normal pseudoplasmodia.

Exercise 13.1 - Sea Urchin Embryology

LEVEL I

Materials

- Prepared slides of sea urchin development
- Microscope
- Male and female sea urchins 16
- 0.5 M KCl
- Depression slides

Procedure

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1. Place a commercially prepared slide of the sea urchin embryo development on the stage of the microscope and locate representative stages in the development of the organism. Draw and label the following stages:

    Primary oocyte with Germinal vesicle
    Fertilized egg with aster
    Two, Four, Eight Cell stages
    Morula (Sixteen cell stage)
    Blastula
    Gastrula

2. Indicate on your drawings the development of micromeres, mesomeres, and macromeres. Also identify the formation of primary mesenchyme and secondary mesenchyme during the formation of the gastrula.
3. Collect a living sea urchin and place it "upside-down" in the top of a small beaker. The mouth should be exposed. With a clean, new needle, inject 1.0 ml of 0.5 M KCl into the tissue just adjacent to the oral opening. The fluid should enter the coelomic cavity, not the gut of the animal.
4. Within a short period of time (several minutes) the sea urchin will discharge its gametes. The gametes will appear as either a yellow collection of eggs, or a white collection of sperm.
   For the eggs, immediately invert the sea urchin so that the eggs are shed directly into a small amount of salt water in the bottom of a new, clean beaker. It is important that the bottom of the sea urchin be in contact with the sea water and that the salt water be kept cool (15° C is ideal). The shed eggs are stable for several hours if kept cool.
   For sperm, the animal should be inverted over a dry beaker, that is with no salt water. Sperm are activated when diluted in sea water. If collected in the dry state and stored in a refrigerator, they will survive for several hours. When diluted, they will last only a matter of minutes.
5. Place a small sample of the eggs in sea water onto a microscope slide and place the slide on the stage of a microscope. Focus on the eggs.
6. With a clean pipette, collect a small drop of the sperm and place it into about 1 ml of salt water in a test tube. Gently stir to mix and activate the sperm, and then transfer a drop of the diluted sperm to the waiting eggs on the microscope stage.

    Do not contaminate the remaining eggs by contact with the sperm.

7. Immediately place a coverslip on the suspension of sperm and eggs and observe. Once fertilization occurs, the edges of the coverslip can be sealed with parafin to prevent drying.
   For long term observation, a diluted sample of eggs should be fertilized and kept at 0° C. Periodically, wet mounts can be made of the developing embryos to examine the progress.
8. Compare the development of the living embryos to the prepared slide stages. 17

Notes

The development of the sea urchin embryo occurs rather rapidly. Fertilization will probably occur before you can get the coverslip on the slide. Within about 1-2 minutes the fertilization membrane will lift off, and the first division should occur within 2-3 hours, depending on the temperature. The embryo will hatch in just over 24 hours and will complete its entire development within 72 hours.

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If close examination of the fertilization process is desired, place the eggs on a slide with a support to keep the coverslip just off the eggs (a second coverslip will do), add a coverslip and focus on the eggs with darkfield illumination. Place a drop of sperm just at the edge of the coverslip while observing the eggs through the microscope. With darkfield illumination the sperm will be visible and minute changes in the cortical region of the egg will be observed as a sperm penetrates the outer membrane.

Exercise 13.2 - Determinants and Fate - *C. elegans* embryos

LEVEL I

Materials

- *Caenorhabditis elegans* cultures on agar plates
- *E. coli* strain OP50
- NG agar plates
- *C. elegans* Ringer's solution
- Microscope (preferrably Nomarski interference or phase)
- Slides and coverslips

Procedure

1. Place a culture of *C. elegans* on the stage of a dissecting microscope and focus on the surface of the agar plate. The cultures contain both small males and the larger hermaphrodites. Locate a specimen of each.
2. For embryological studies, select a large hermaphrodite and identify the various organs within the worm. With a wooden applicator, select a hermaphrodite and transfer it to a slide containing a drop of Ringer's solution. Make a wet mount. 18
   The coverslip should hold the worm down, but will allow it to continue moving. It will normally be very active at first, but then quiet down. Since the worm can only flex its body in a dorsal/ventral plane, it will also normally be on its side.
3. Place the slide on a microscope 19 and locate the worm in the field of view. Identify the eggs and developing embryos within the ovary of the worm. Careful adjustment of the light will be necessary. As the slide dries out, it will be necessary to add a drop of Ringer's to the edge of the coverslip.
4. Draw the various stages of development and label each cell according the the scheme in Figure 13.6.

Optional

Select several gravid worms (hermaphrodites) and transfer them to a fresh plate of NG agar pre-seeded with a lawn of *E. coli*. Allow the worms to lay eggs on the agar.
After the eggs are sown, use Ringer's solution to rinse the adult worms from the agar plate. Any remaining adults should be picked off the agar, but the eggs will remain stuck in the agar. This process will nearly synchronize the eggs and their development can then be observed over time. L1 larva can be washed from the plates in about two hours and the subsequent development of these larva monitored as a synchronous population.

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Exercise 13.3 - The Hematopoietic System

LEVEL I

Materials

- Prepared slide of bone marrow
- Microscope

Procedure

1. Examine the bone marrow slide to identify the erythrocytic series: proerythroblast, basophilic erythroblast, polychromatophilic erythroblast, normoblast, reticulocyte and mature erythrocyte. Draw and label each cell type.
2. Draw and label the granulocytic series: myeloblast, promyelocyte, myelocyte, metamyelocyte, granulocyte with a band- shaped nucleus, and the mature granulocyte (neutrophil, eosinophil, basophil).
3. Draw and label the megakaryocytic series: megakaryoblast, megakaryocyte and platelet. 20

Exercise 13.4 - Photomorphogenesis

LEVEL I

Materials

- *Pteridium aquilinum* spores (Bracken fern)
- Agar plates of Knudson media 21
- Cellophane filters (red, blue and green 22)
- Dissecting microscope
- 1% (v/v) Tween 80 (Polyoxyethylene sorbitan mono-oleate)

Procedure

1. Place 10 ml of Tween 80 into a capped test tube and add about 1-2 mg of dry fern spores. Cap the tube and shake the vial to wet the spores.
2. Obtain eight petri plates containing a minimum balanced salt media. Pipette 1.0 ml of the spore suspension onto the surface of each plate and swirl the plate to evenly distribute the spores. Place the lids on the petri plates and seal the plates with tape running completely around the edge. 23
3. Wrap the plates in pairs of red, blue or green cellophane. Leave two plates with no wrapping. Place the plates under a light source (window sill or fluorescent "Gro-lux" with a 12/12 (12 hrs. light, 12 dark) light regime.
4. Monitor the spores daily by observation with a dissecting microscope. When they begin to germinate, monitor with the low power of a regular microscope (or inverted, if the agar is not too thick).
   The spores will extend a rhizoid in about 5 days and begin cell division within 7-8 days. The first divisions are then crucial to the development of the gametophyte shape. Continue to monitor the division planes for a period of two to three weeks.

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5. Note the number of spores that germinate and calculate the percent germination. As the prothalli grow, note the position and direction of each cell division. Sketch each stage in the development of the fern gametophyte.

Optional

Fix gametophytes periodically with acid-alcohol and prepare the gametophytes for histological staining of the chromosomes. Note the presence of mitotic figures and identify the poles of each cell division. Based on the location of the division planes, predict the direction for spindle fiber growth and hypothesize a mechanism for controlling the direction of cell division (and therefore the morphology of the gametophyte). Devise an experiment to test your hypothesis.

One can do more advanced study of morphology by combining the growth of the gametophyte with the presence of mitotic inhibitors or chromosome damaging agents (Chapter Ten). Finally, the spores can be subjected to x-ray exposure (40-60,000 roentgens) and the morphology studied. As the fern "tumors" develop, the planes of division will appear randomly, giving rise to a 3-dimensional growth rather than the typical 2-dimensional gametophyte.

Exercise 13.5 - *D.discoideum* Growth on Agar

LEVEL II

Materials

- *Dictyosteleum discoideum* grown axenically on agar plates
- Petri plates with SM agar media
- Broth culture of *Klebsiella aerogenes*
  or *E. coli* (non-mucoid variety)
- Wire loop for transfers
- Microscope, slides, coverslips

Procedure

1. Select a petri plate with SM agar media, a broth culture of and a culture of D. discoideum containing fruiting bodies.
2. Inoculate an agar plate with a thin layer of the bacteria. Select a fruiting body from the fungal culture and place it in the center of the petri plate containing the bacterial suspension.
3. Continue to monitor the growth of the fungus for the next week (make daily observations - new fruiting bodies will appear in about 4 days).
   Note the initial germination of the spores from the fruiting bodies, the subsequent growth and the increased number of amoeba in the culture. As the culture begins to dry, the amoeba will aggregate and form the migrating slug. The slugs will migrate around the plate, consuming the bacteria as food.
   Eventually, the slug will anchor itself to the agar and undergo a process known as culmination. During this stage, the cells in the anterior of the slug will begin differentiating into pre-stalk cells, while those in the rear will develop into pre-spore cells.
4. Sketch each stage in the development of the fungus and note the time of each stage appearance within

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the culture.

Optional

Periodically remove a sample of the cells or slug and fix with Carnoy fixative. Place the cells on a slide and allow to air dry. Stain with a a basophilic dye and note any alterations in the nuclear material during each phase. In particular, note the presence and size of the nucleoli, and the degree of basophilia demonstrated by the cytoplasm.

Exercise 13.6 - Suspension cultures of *D. discoideum*

LEVEL II

Materials

Axenic culture selected for suspension growth (e.g. *D. discoideum* Strain AX-3 24)

Cultures supplemented with 10^{-3} M cAMP25

Fresh flasks of growth media, with and without cAMP

MES-PDF containing 10 mM EDTA (Optional)

Procedure

1. Collect amoeba from the normal media cultures. Examine the culture for the presence of the amoeba and note the stage of their development (i.e. individual amoeba, aggregates, slugs).
2. Select amoeba from the cAMP treated culture and compare the amoeba to those grown in the absence of the nucleotide.
3. Using a pipette, transfer an aliquot of the amoeba previously grown in the absence of cAMP to fresh media without cAMP. Establish a second culture by transferring the same amoeba to media containing cAMP. Follow the techniques outlined for the transfer of eukaryotic cells in Chapter Twelve.
4. Prepare a growth curve for each of the amoeba subcultures (i.e. amoeba grown in the presence and absence of cAMP).
5. Note on the growth curve the times for any observed differentiation.

Optional

When the amoeba aggregate, the position the cells have within the slug determines its ultimate fate. If a cell is in the anterior of the slug, it will become part of the subsequent stalk. If in the posterior, the same cell would become part of the spore forming body.
The pseudoplasmodia are attracted toward a strong light, and this taxis has been used to orient slugs and consequently separate cells on the basis of their position.
Plate Ax-3 cells onto Millipore filters for development (5×10^6 cells/cm^2). At the finger stage, shake the cells from the filters and dissociate them into single cells by vigorous pipetting in cold

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MES-PDF containing 10 mM EDTA. Shake the suspension for 3 hours under one of the following conditions:

No added cAMP @230 RPM
1 mM cAMP, with hourly additions to 100 microM, 230 rpm
No added cAMP @ 70 RPM

The fast shaking cultures will remain as predominantly single cells while those in the slowly shaking culture will aggregate. 26
Harvest the cells by centrifugation and analyze for size of the aggregates as well as formation of the multicellular structures.

Exercise 13.7 - Isolation of Fruiting Bodies without slugs

LEVEL III

Materials

- 24 suspension cultures of *D. discoideum*
- Clinical centrifuge and tubes
- Black Millipore filters (AABP47SO)
- Cellulose pads for Millipore filters
- LPS buffer

Procedure 27

1. Cultures grown for 24-30 hours (agar or suspension) are washed free of bacteria by several centrifugations in fresh, cold water (2 minutes @ 300 Xg each).
2. Apply the cells at a density of 6 X 10^6 cells per cm^2 to a water washed black Millipore filter supported by two cellulose absorbent pads previously saturated with LPS Buffer.
3. Allow the cells to develop in the dark at 22° C in a humidity saturated chamber.
4. Periodically withdraw a sample of the amoeba and sketch the development of the aggregates. Note in particular the induction of synchronous fruiting without the formation of migrating slugs.

Exercise 13.8 - Prestalk vs Prespore Effect

LEVEL III

Materials

- Agar cultures of *D. discoideum*
- 20 mM Na-K Phosphate buffer, pH 7.0
- 20 mM Na-K Phosphate buffer plus 20 mM EDTA, pH 7.0
- Hypodermic syringe with 16 gauge needle
- Nylon mesh

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- 70% v/v Percoll in 20 mM EDTA, 5 mM MES, pH 7.0
- Preparative centrifuge, rotor and tubes

Procedure 28

1. Collect migrating slugs by washing them from the agar with cold 20 mM Na-K Phosphate Buffer, pH 7.0. A bent glass rod may be used to assist in dislodging the slugs.
2. Wash the slugs once with the same buffer and resuspend in 20 mM Na-K Phosphate Buffer containing 20 mM EDTA.
3. Pipette the slugs up and down through a 16 gauge needle for about 5 minutes to dissociate the cells. 29
4. Pass the suspension of cells through a fine nylon mesh to remove clumps, wash with the EDTA buffer and resuspend in 2-3 ml of fresh EDTA buffer. Collect the cells by centrifuging for 2 minutes at 300 xg. Resuspend the cells to make a final concentration of 4 X 10⁸ cells per ml of EDTA buffer.
5. Form two Percoll gradients by placing 12 ml of Percoll into two centrifuge tubes and centrifuging at 21,000 xg for 40 minutes at 4° C.
6. Layer 250 µl of the cell suspension onto a preformed Percoll gradient. Either distribute the cells between the two gradients, or use one tube as a balance in the centrifuge.
7. Centrifuge the amoeba in the preformed Percoll gradient at 13,000 xg at 15° C for 3 minutes.
8. Collect and identify the bands of cells. Wash the cells free of Percoll with EDTA buffer.

Notes

Once separated on Percoll, the cells can be analyzed for any number of activities. One of the principal analyses is for the production of specific gene products which would indicate DNA control of differentiation. The effect of differential gene activity can be studied by extracting RNA from the prestalk and prespore cells with subsequent analysis of the RNA.
Since the ultimate gene product is a protein, several investigators have identified and isolated proteins characteristic of prestalk and prespore differentiation. By producing antibodies to those proteins, immunofluorescent techniques may be applied to the detection of early differentiation. Figure 13.7 demonstrates such an analysis applied to prestalk/prespore cells.

Endnotes

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16. *Unfortunately there is no external means of determining gender of the sea urchin. When Strongylocentrotus purpuratus is induced to shed gametes, the eggs that are released will be a yellow color, while the sperm is white. Thus, the gender is determined via the gametes that are released.*
17. *For assistance in your drawings, excellent photographs of early embryology are found in Mathews, Wills W. Atlas of Descriptive Embryology, 3rd Ed. Macmillan, New York, 1982.*
18. *If long term obsrvation is desired, a special slide needs to be made. Place masking tape on a slide, with a hold cut in the middle. Put a small drop of liquified NG agar on the slide and place a siliconized coverslip immediately on the agar. When the agar solidifies, remove the coverslip. Just prior to use, place a very small amount of E. coli on the agar, transfer a worm to the agar, and place a coverslip over the agar and the tape. Once the worm takes to eating the bacteria, it will settle down and can be readily observed for hours.*
19. *Use Normarski interference optics if available. Otherwise alternate between bright field and dark field views.*
20. *For a thorough review of the hematopoietic system, refer to Junqueira, L.C., J. Carneiro, and A.N. Contopoulos. Basic Histology. Lange Medical Publications, Los Altos, 1975.*
21. *Although Knop's is more commonly used, the author has used Knudson's for many years. Any minimal plant media with minor and major salts will work. The critical factor is low pH, with an ideal of about 5.5. If organic elements are added (such as sucrose) then the media must be sterilized to prevent the growth of fungi. Sterility is not required if the organic elements are eliminated, and the fern spores will germinate extremely well. In fact they will germinate reasonably well on the surface of a clay flower pot that is kept moist with plain tap water.*
22. *Photomorphogenesis depends on the quality of these filters. Many lightly colored filters allow*

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transmission of significant amounts of light other than the color they appear to be. To check, cut a small piece of the cellophane and tape it to a spectrophotometer cuvette. Run an absorption spectrum on the cellophane to identify the wavelengths which are transmitted.

23. *Alternatively, the agar media can be put in baby food jars, the spores placed on the agar, the tops sealed with plastic wrap and held in place by a rubber band. This system will allow the gametophytes to develop completely over periods greater than 6 months, and will even support the early growth of the sporophyte. By contrast, the petri plates are useful only for about two weeks.*
24. *Firtel, R.A. and J.T. Bonner. "Characterization of the genome of the cellular slime mold Dictyostelium discoideum." J. Mol. Biol. 66:339-361. 1972. This culture has the advantage of easy administration of cAMP and differentiation can be controlled to some extent by the speed with which the amoeba are shaken. Slow speeds allow the amoeba to form aggregates, while higher speeds impede this formation.*
25. *Further differentiation can be had by "pulsing" the cultures with cAMP. In the normal aggregation and development of the slime mold, the cells do not continuously secrete cAMP, but secrete it in increasing measured doses.*
26. *Reymond, et al. Cell 39:1984.*
27. *Ratner, D. and W. Borth. "Comparison of Differentiating Dictyostelium discoideum Cell Types Separated by an Improved Method of Density Gradient Centrifugation". Exper. Cell Research 143 (1983) p. 1-13.*
28. *Ratner, D. and Borth W. 1982. "Comparison of differentiating Dictyostelium discoideum cell types separated by an improved method of density gradient centrifugation." Exp. Cell Res. 143;1-13. 1983.*
29. *If the slugs do not readily dissociate, 12.5 mM 2,3-dimercaptopropanol and 1 mg/ml pronase may be added.*

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Chapter 14: Nucleic Acids

Introduction

The previous exercise dealt with differentiation, and specifically with the events leading to differential gene activity. In molecular terms, this process involves regulation using DNA as a primer molecule for the selective synthesis of RNA. DNA primed RNA synthesis is termed "transcription."

Transcription is a complex series of reactions which involve the use of an RNA polymerase enzyme, known as transcriptase. If the reaction occurs in reverse, that is with an RNA primer synthesizing DNA, the process is known as reverse transcription, and the enzyme is reverse transcriptase. This latter process is important for RNA virus replication in general, and is most significant when examining oncornagenic virus. Oncornagenic virus are RNA-containing virus that are also causal agents for some forms of cancer (primarily in birds). Reverse transcription is also important in the development of amphibians, and in the process of gene amplification.

Exercise 14.1 - Extraction of DNA from Bovine Spleen

LEVEL I

Materials

- Bovine spleen
- Saline Citrate Buffer (SSC)
- Chilled blender
- Refrigerated preparative centrifuge
- 2.6 M NaCl
- 95% (v/v) ethanol

Procedure

1. Weigh out approximately 15 grams of frozen bovine spleen. Record the exact weight for future reference.

   Weight of the liver _____ gm

2. Drop the pieces of spleen one at a time into a chilled blender containing 150 ml of cold citrate-saline buffer (SSC). Continue to homogenize the tissue until it is thoroughly macerated. Do not over-homogenize and allow the contents to warm up. Proper procedure should take about 30-60 seconds of blending.
3. Pour the homogenate into nalgene centrifuge tubes and centrifuge at 4,000 xg for 15 minutes at 4° C.
4. Decant the supernatant and discard.

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The supernatant contains most of the materials that are soluble in physiological buffer. RNA, protein and many carbohydrates are found in this portion. The pellet contains most of the DNA, but it is complexed and in the form of DNP. The pellet also contains any cell debris and unbroken cells resulting from the homogenization.

5. Resuspend the pellet in about 20 ml. of saline/citrate buffer by gently stirring with a glass rod. If the pellet is packed hard and will not disperse easily, you may use a vortex mixer to aid in the dispersion.
6. Recentrifuge as in step 3. Discard the supernatant.
7. Add 20 ml. of cold 2.6 M NaCl. Break up the pellet with a glass rod, close the centrifuge tube with a tight fitting cap and shake vigorously. DNA is soluble in cold NaCl and will also dissociate from the protein. Pour off the liquid portion from this procedure and save for next step. Add another 20 ml. of cold 2.6 M NaCl and shake vigorously. Continue to do this for two more extractions. It is important that the salt be kept cold (use an ice bath) and that the shaking be vigorous. Breaking the pellet with a glass rod may also help.
8. Combine all four extractions from above and centrifuge at 20,000 xg for 20 minutes. This will pellet the insoluble proteins.
9. Pour the supernatant carefully into a liter beaker and slowly add 2.3 volumes of cold 95% ethanol allowing it to pour down the side of the beaker and layer on top of the aqueous supernatant.
10. Collect the DNA by gently stirring the mixture together.
    DNA, if it is highly polymerized, will "spool" onto a clean glass rod as the salt solution is mixed with the alcohol. It can then be removed from the solution in the beaker, washed twice with cold 70% ethanol and placed into 70% ethanol or lyophilized for storage.

Notes

DNA spooled by this procedure is impure. Before it can be used for further analysis, care must be taken to remove contaminating protein, RNA, and carbohydrate. There are a number of means to accomplish this, most involving either enzyme digestions (pronase, amylase, and RNAse) or differential salt solubility or combinations of these techniques (Exercise 14.2).

Exercise 14.2 - Purification of DNA

LEVEL I

Materials

- Spooled DNA from Exercise 14.1
- SSC buffer
- Pancreatic Ribonuclease A (100 µg/ml)
- Pronase
- Sodium lauryl sulfate (SLS)
- Sodium perchlorate
- Chloroform:isoamyl alcohol (24:1)
- 95% and 70% (v/v) ethanol
- Refrigerated centrifuge, rotor and tubes

Procedure 1

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1. Decant off the alcohol, and dissolve the extracted DNA in 30 ml. of diluted SSC buffer (0.1 X SSC) in a 125 ml. erlenmeyer flask. This will require some time, as polymerized DNA dissolves slowly. Gentle swirling of the material will help.
2. Add pancreatic ribonuclease A to a final concentration of 100 micrograms/ml and agitate slowly at 37° C for 1 hour.
3. Add pronase to a final concentration of 50 micrograms/ml and again agitate slowly at 37° C for another hour.
4. Add sodium lauryl sulfate (SLS) 2 to make a 1% concentration (w/v) and sodium perchlorate to a final concentration of 1 M. Agitate for 30 minutes at room temperature.
5. Extract the solution with chloroform:isoamyl alcohol (24:1 v/v) by adding an equal volume and shaking virogously for at least 15 minutes.
6. Place the solution into appropriate centrifuge tubes and centrifuge for 5 minutes at 800 xg and 4° C.
7. Remove the upper aqueous phase, add 2.3 volumes of 95% cold ethanol and respool the DNA from this solution onto a glass rod.
8. Wash the spooled DNA twice with cold 70% ethanol and store for future analysis.

Exercise 14.3 - Characterization of dna

LEVEL I

Materials

- DNA sample
- SSC buffer
- UV spectrophotometer 3 and quartz cuvettes

Procedure

1. Dissolve a small quantity of your extracted DNA in 3.0 ml of 0.1X SSC.
2. Turn on and blank a UV spectrophotometer at 220 nm (use 0.1X SSC as the blank). Determine the absorbance of your sample DNA at 230 nm.
3. Change the wavelength to 230 nm, reblank the spectrophotometer and measure the absorbance of the sample at 230 nm.
4. Increment the wavelength by 10 nm and repeat blanking and measuring the absorbance until readings are taken through 300 nm.
5. Compute the absorbance ratio 260 nm to 280 nm. Pure DNA (without protein or RNA) will have a 260:280 absorbance ratio of 1.85. RNA will have a 260:280 ratio of 2.0.
6. Plot the absorbance spectrum of your sample and indicate the 260:280 ratio, as well as the amount of protein contamination on the graph.

Wavelength	**Absorbance**
220	
230	

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240	
250	
260	
270	
280	
290	
300	

Exercise 14.4 - DNA - Dische Diphenylamine Determination

LEVEL I

Material

- Lyophilized DNA standard
- Sample DNA
- SSC
- Dische diphenylamine reagent
- Spectrophotometer

Procedure

1. Weigh out 15.0 mg of commercial lyophilized DNA and prepare a stock solution of 3.0 mg/ml by dissolving the DNA in 5.0 ml of SSC. This material will be used to prepare a standard curve for the diphenylamine reaction.
   Note that lyophilized, highly polymerized DNA is extremely slow to go into solution. It will require preparation at least one day in advance of lab with constant shaking.
2. Prepare a series of known standard solutions by serially diluting the stock solution of DNA. Set up a series of test tubes containing 2.0 ml of SSC each. Pipette 2.0 ml of stock solution into tube #1, mix and pipette 2.0 ml of the resulting mixture into tube #2 and so on. This will yield a series of tubes containing 1.5, 0.75 and 0.375 mg/ml of DNA. Your original stock solution is 3.0 mg/ml and SSC should be used for the blank.
3. Remove and discard 2.0 ml of the final dilution. To each of the five tubes containing in step 2 (each should contain only 2.0 ml), add exactly 4.0 ml of Dische diphenylamine reagent and mix well.

   This Reagent contains glacial acetic acid. It is caustic and should be handled with care.

4. Place a marble on the top of each test tube (it should not fall into the test tube, as it will act as a reflux to prevent evaporation, while allowing for pressure changes). Place the tubes in a boiling water bath for 10 minutes, remove from the bath and immediately immerse in an ice bath to cool.
5. Turn on a spectrophotometer and adjust the wavelength to 650 nm. Use the tube containing no DNA from step 2 to blank the instrument and measure the absorbance of each of your standards.

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Plot the absorbance against DNA concentration, perform a linear regression of the data and compute the extinction coefficient.

6. Dissolve your extracted or sample DNA in 10 ml of SSC. Make serial dilutions of 1/10, 1/100 and 1/1000 with SSC. Measure the absorbance of your extracted or sample DNA dilutions and calculate the concentration of DNA in the sample. Use the dilution which gives an absorbance in the 0.1 to 1.5 range.

Exercise 14.5 - Melting Point Determination

LEVEL II

Materials

- DNA
- SSC
- UV spectrophotometer (preferably with temperature control)

Procedure [4]

1. Dissolve your DNA preparation in SSC to give a final concentration of approximately 20 µg DNA/ml.
2. Place the dissolved DNA in an appropriate quartz cuvette along with a second cuvette containing SSC as a blank.
3. Place both cuvettes into a dual beam temperature regulated UV spectrophotometer and measure the absorbance of the sample at 260 nm at temperatures ranging from 25° C to 80° C. Continue to increase the temperature slowly and continue reading the absorbance until a sharp rise in absorbance is noted.

   Alternatively:

   a. Place the cuvettes into a waterbath at 25 ° C and allow to temperature equilibrate. Remove the blank, wipe the outside dry and rapidly blank the instrument at 260 nm. Transfer the sample to the spectrophotometer (be sure to dry and work rapidly) and read the absorbance.

   b. Raise the temperature of the bath to 50° C and repeat step a.

   c. Raise the temperature sequentially to 60° C, 65° C, 70° C, 75° C and 80° C and repeat the absorbance measurements.

   d. Slowly raise the temperature above 80° and make absorbance measurements every 2° until the absorbance begins to increase. At that point, increase the temperature, but continue to take readings at 1° C intervals.

4. Correct all of the absorbance readings for solvent expansion relative to 25° C. [5] List the corrected values as A_t
5. Plot the value of A_t / A_{25} vs temperature and calculate the midpoint of any increased absorbance. This midpoint is the melting point (Tm) for your DNA sample.
6. Calculate the GC content of your sample using the formula

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Percent of G + C = k(Tm -69.3) x 2.44

Notes

Single strand DNA absorbs more UV light than double strands. Moreover, double strands can be separated by heat (melted) and the temperature at which the strands separate (Tm) is related to the number of guanine-cytosine residues (each having three hydrogen bonds as opposed to the two in adenine-thymine). This has led to the development of a rapid test for an approximation of the GC/AT ratio using melting points and the change in UV $_{260}$ absorbance (known as "hyperchromicity" or "hyperchromatic shift"). Of course, the separation is also dependent upon environmental influences, particularly the salt concentration of the DNA solution. To standardize this, all Tm measurements are made in SSC buffer. DNA melts between 85° and 100° C in this buffer (as opposed to 25° C in distilled water).

Exercise 14.6 - CsCl - Density Separation of DNA

LEVEL II

Materials

- DNA
- CsCl
- 0.3 N NaOH
- 0.2 M Tris-HCl buffer, pH 7.0
- Ultracentrifuge and rotor
- UV spectrophotometer and cuvettes

Procedure

1. Determine the G+C content of the sample DNA (Exercise 14.5)
2. Once the G+C content is determined, the bouyant density of the DNA can be determined from the formula:

   p = 1.660 g/cm^3 + 0.098 x (G+C fraction)

   Using the bouyant density and the CsCl Table in Appendix F, determine the concentration of CsCl salts to use for dissolution of the DNA.

3. Dissolve approximately 100 micrograms of DNA in 4.2 ml of the appropriate CsCl solution in 0.3 N NaOH.
4. Load the dissolved DNA/CsCl solution onto a centrifuge tube suitable for a 4.2 ml sample and speeds of 30-40,000 RPM (Beckman SW39 rotor, or equivalent).
5. For the Beckman SW39 rotor, centrifuge the material at 35,000 RPM for 65 hours at 22° C.
6. Collect the fractions in 0.1 ml steps.
7. Add 0.2 M Tris-HCl, pH 7.0 to each fraction and measure the A$_{260}$ for each fraction. If available, a continuous flow system using a fraction collecting device may be used.

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Exercise 14.7 - Phenol Extraction of rRNA (Rat liver)

LEVEL II

*** READ THROUGH ALL CAUTIONS BEFORE TRYING THIS EXPERIMENT ***

Materials

- Rat liver (fasted rat)
- Liquid nitrogen
- p-Amino-salicic acid
- Phenol mixture
- Homogenizer or blender6
- Refrigerated preparative centrifuge
- NaCl
- 95% and 70% (v/v) ethanol

Procedure

1. Obtain a rat which has been fasted for 24 hours (to remove glycogen from the liver), decaptitate, exsanguinate and remove the liver as rapidly as possible.
2. Weigh the liver, being careful not to allow the it to dehydrate.
3. Immediately drop the liver into a container of liquid nitrogen.

   CAUTION: Liquid nitrogen will cause severe frostbite!

4. Using the weight of the liver as an indication of the volume (1 gm of liver equivalent to 1 ml), add 15 volumes of freshly prepared 6% para-amino-salicylate (pAS) to a chilled blender or homogenizer.
5. Add an equal volume (equal to the pAS) of phenol mixture to the blender and turn on the blender for a short burst to mix the pAS and phenol.

   CAUTION: Phenol is extremely caustic.

   Phenol causes severe skin burns, yet it is a local anesthetic. You will be unaware of the burn at first, except for tell-tale discoloration of the skin and blisters. You will become aware of the burn as the anesthetic properties wear off. Phenol also readily dissolves most countertops and all rubber compounds.

   CLEAN UP ALL SPILLS IMMEDIATELY! NOTIFY YOUR INSTRUCTOR OF ANY SPILLS AFTER YOU HAVE THOROUGHLY RINSED AND WASHED AWAY ANY MATERIALS IN CONTACT WITH YOUR SKIN.

6. Stop the blender and add the frozen liver (handle the liver with long forceps, or tongs). Blend the entire mixture (pAS, phenol and liver) for 30 seconds at full speed. Do not blend for longer periods or you will sheer the RNA.
7. Carefully transfer the homogenate to a beaker and continue to stir the mixture for 10 minutes at room temperature.

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8. Transfer the homogenate to nalgene centrifuge tubes and centrifuge the mixture at 15,600 xg at 4° C for 20 minutes.
9. Remove the centrifuge tubes and carefully separate the upper aqueous layer from the lower phenol layer. Take care that none of the white interphase material is mixed into the aqueous layer. The upper layer can most efficiently be removed by using a large hypodermic equipped with a long, large bore, square tipped needle. Should some of the interphase material be stirred into the aqueous phase, it will be necessary to repeat step 8.
10. Measure the volume of the aqueous layer and discard the phenol layer and interphase material.
11. Add 3.0 grams of NaCl per 100 ml. of aqueous phase and stir until dissolved.
12. Add 0.5 volumes of phenol mixture to the aqueous phase, place into a suitable flask and shake vigorously for about five minutes. Recentrifuge as in step 8 above, but for 10 minutes.
13. Separate the aqueous phase and add 2.3 volumes of cold 95% ethanol. Allow the mixture to stand in the freezer until a precipitate forms.
14. Collect the RNA precipitate by centrifugation, wash once in 70% ethanol and store in 70% ethanol at 0-5° C.

Notes

Knowledge of transcription is based on our ability to extract "native" or functional RNA molecules from cells, with subsequent use of those molecules "in vitro." One of the earliest methods for this type of analysis is a phenol-detergent extraction of RNA 7 coupled with separation of the various sized molecules of RNA with centrifugation in a gradient.

This basic procedure remains useful today, although there have been myriad additions and alterations to the procedure using a host of extraction techniques and separation procedures (such as electrophoresis or column chromatography).

For the purposes of introduction to the technique, this exercise extracts RNA from rat liver using a phenol extraction which yields predominantly rRNA and tRNA. There is some mRNA present, but it is variable and should be considered as a background contaminant. There is also a good portion of sRNA caused by sheering of the RNA during homogenization, and by enzymatic digestion by RNAase during the extraction.

Exercise 14.8 - Spectrophotometric Analysis of rRNA

LEVEL I

Materials

- RNA
- UV spectrophotometer and cuvettes
- Alkaline distilled water

Procedure

1. Dissolve 10 mg of commercial RNA in 250 ml. of slightly alkaline distilled water. Use a volumetric flask

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and proper analytical technique. This will give a standard solution of 40 micrograms RNA/ml.

2. Prepare a series of dilutions so that you have 40, 20, 10, 5 and 2.5 micrograms of RNA per ml.
3. Turn on a UV spectrophotometer and adjust the wavelength to 260 nm. Use the alkaline water to blank the spectrophotometer at 260 nm.
4. Read the A_{260} of each of the standards. Plot the A_{260} vs the concentration of RNA and calculate the extinction coefficient.
5. Dissolve your isolated, precipitated RNA in 10.0 ml of alkaline water. Prepare a serial dilution for 1/10, 1/100, 1/1000 and 1/10000. Measure the absorbance of each at 260 nm and, using the dilution which gives a reading between .1 and 1.5 absorbance units, compute the concentration of RNA in your sample.

Exercise 14.9 - Orcinol Determination of RNA

LEVEL I

Materials

- RNA
- Alkaline distilled water
- Acid-orcinol reagent
- Boiling water bath
- Spectrophotometer and cuvettes

Procedure

1. Prepare a series of serially diluted RNA standards as in Exercise 14.8, but having a range from 1.0 mg/ml down to 0.125 mg/ml.
2. Prepare a serial dilution of your sample RNA as in Exercise 14.8.
3. Place 3.0 ml of each standard and 3.0 ml of each serial dilution of the sample RNA into separate test tubes. Place 3.0 ml of alkaline water in a separate tube.
4. Add 3.0 ml of acid-orcinol reagent to each tube and mix well.
5. Add 0.3 ml of alcohol-orcinol reagent to each tube and mix well.
6. Place the tubes in a boiling water bath for 20 minutes with marbles placed on top of each tube to prevent evaporation. Cool the tubes by immersion in an ice bath at the end of the 20 minute period.
7. Turn on a spectrophotometer and adjust the wavelength to 660 nm. Blank the spectrophotometer with the alkaline water/orcinol tube. Measure the A_{660} of each of the remaining standards and diluted samples.
8. Plot the absorbance of the standards against the known concentrations. Calculate the extinction coefficient, and calculate the concentration of RNA in your sample. Use the dilution yielding an absorbance between 0.1 and 1.5 absorbance units.

Exercise 14.10 - Sucrose Density Fractionation

LEVEL II

Figure 14.2 Sucrose gradient distribution of RNA

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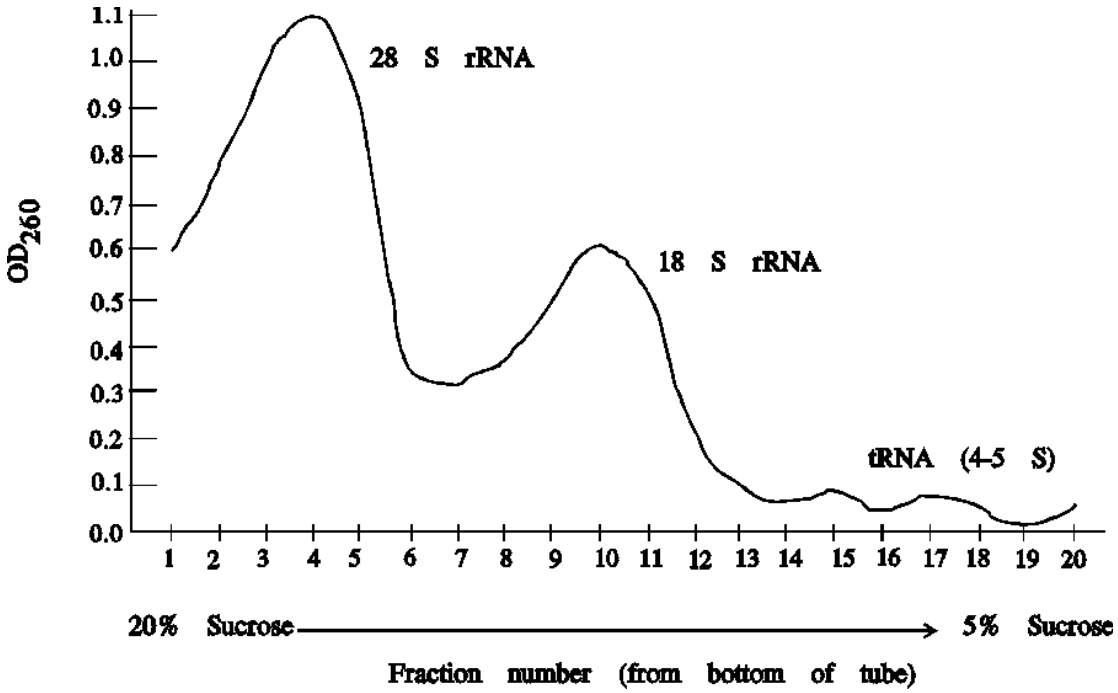

Materials

- 10% and 40% (w/v) Sucrose 8
- 0.02 M sodium acetate, pH 5.1, containing 0.1 M NaCl and 1 mM
- EDTA
- RNA
- Ultracentrifuge, swinging bucket rotor and tubes
- Centrifuge tube fractionating device
- UV spectrophotometer

Procedure

1. Refer to Chapter Three for details of fractionation, and form a 10-40% linear sucrose gradient in a nitrocellulose tube.

    a. Close all valves on the gradient device.

    b. Place exactly 15 ml.9 of 40% sucrose in the right chamber and 15 ml. of 10% sucrose in the left chamber of the gradient device.

    c. Open the flow from the right chamber to the centrifuge tube. Be sure there is a tube in place, that the flow is directed down the inside of the tube and that the magnetic stirrer is functioning.

    d. Immediately open the valve on the left chamber and insure that sucrose is flowing from left to right, thereby diluting the 40% sucrose with 10% sucrose.

    e. Allow the flow to continue until all of the sucrose enters the centrifuge tube.

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2. Dissolve the RNA in 0.02 M sodium acetate solution to yield a final concentration of 250 µg/ml. The low pH of the solution helps to inhibit RNAse, while the salts will keep the RNA from forming large polymeric aggregates. 10
3. Carefully layer 2.0 ml of the dissolved RNA onto the top of a sucrose gradient. This should be done by slowly allowing the solution to run down the side of the tube and onto the gradient. Be careful not to disturb the gradient.
4. Load the ultracentrifuge with the prepared tubes and centrifuge for the equivalent of 18,700 RPM for a Beckman SW27 rotor, for 20 hours at 4° C (Refer to Appendix F).
5. At the completion of centrifugation, remove the tubes and fractionate the contents into 1.0 ml fractions.

    a. Insert the nitrocellulose centrifuge tube into the plexiglass holder, but be careful NOT to puncture the bottom.

    b. Have a series of 30-35 test tubes ready to accept the effluent from the tubes. Each tube should be labeled and kept in order.

    c. Push down on the centrifuge tube (Gently!) in order to puncture the bottom of the tube and immediately begin to collect the effluent.

    d. Count the drops that fall from the device, and place 40 drops into each tube.

6. Using a UV spectrophotometer 11 and microcuvettes, read the A_{260} of each fraction. Calculate the amount of RNA in each fraction.
7. Plot the concentration of RNA in each fraction against the fraction number. Based on the density of sucrose in each fraction, compute the density of the RNA in that fraction. Based on the relative size (greater density) of the RNA, determine the nature of the fractions (i.e. rRNA, tRNA).

Exercise 14.11 - Nucleotide Composition of RNA

LEVEL II

Materials

- RNA sample
- 1 N and 0.1 N HCl
- Boiling water bath
- Whatman #1 filter paper (for chromatography)
- Chromatography tank
- 20 µl micropipette
- Acetic acid: butanol: water (15:60:25) solvent
- UV light source
- UV spectrophotometer

Procedure

1. Place a portion of your RNA sample (approximately 40 mg, hydrated) into a heavy walled pyrex test tube.

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Add 1.0 ml of 1 N HCl and seal the tube.

2. Heat the tube in a boiling water bath for 1 hour.
3. Cool the tube, open it and place the contents into a centrifuge tube. Centrifuge the contents at 2,000 RPM in a clinical centrifuge to remove any insoluble residue. The supernatant contains your hydrolyzed RNA.
4. Prepare Whatman filter paper No. 1 for standard one- dimensional chromatography. 12
5. Using a micropipette, spot 20 µl of your hydrolyzate onto the paper, being careful to keep the spots as small as possible (repeated small drops are better than one large drop). Allow the spots to completely dry before proceeding.
6. Place the paper chromatogram into your chromatography tank and add the solvent (acetic acid:butanol:water). Allow the system to function for an appropriate time (approximately 36 hours for a 20 cm descending strip of Whatman #1). Remove the paper and dry it in a circulating air oven at 40° C for about 2 hours.
7. Locate the spots of nucleotides by their fluorescence under an ultra-violet light source. Expose the paper chromatogram to a UV light source and outline the spots using a light pencil. The order of migration from the point of origin is guanine (light blue fluorescence), adenine, cytilic acid and finally, uridylic acid.

   Do not look directly at the UV light source. Use a cabinet designed to shield from harmful UV radiation.

8. After cafefully marking the spots, cut them out with scissors and place the paper cut outs into separately labeled 15 ml conical centrifuge tubes. Add 5.0 ml of 0.1 N HCl to each tube and allow the tubes to sit for several hours to elute the nucleotides from the paper.
9. Pack down the paper with a glass rod (centrifuge in a clinical centrifuge if necessary) and remove an aliquot of the liquid for spectrophotometric assay.
10. Measure the absorbance of each of the four nucleotides at the indicated UV wavelength (having first blanked the instrument with 0.1 N HCl).

Base	Wavelength	Molar Extinction Coefficient
Guanine	250 nm	10.6
Adenine	260 nm	13.0
Cytidylic acid	280 nm	19.95
Uridylic acid	260 nm	9.89

11. Use the molar extinction coefficients to determine the concentration of each base in the sample. Calculate the percent composition of each base, and the purine/pyrimidine ratio.

Exercise 14.12 - Other studies

LEVEL III

Once DNA has been purified and analyzed, there are many procedures that can be followed. Hydroxyapatite columns can easily separate single stranded DNA from double stranded DNA and are used extensively in gene transplant work. This can be appended to the formation of DNA-RNA

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hybrids, whereby an mRNA is re-annealed to DNA, all single strands eliminated and the resulted hybrid re-separated. The remaining DNA strand is the gene for that mRNA (although not complete, some start, stop and housekeeping codes will be missing).

If one has a sufficient quantity of RNA's, it is thus possible to create a DNA "library" for these genes. Modern approaches will use the mRNA to synthesize DNA rather than isolating it through hybridizion. DNA synthesized from RNA is known as cDNA, which presents us with powerful "libraries" for analysis of cell function at the gene level.

Once obtained, the DNA can be readily sequenced, although the techniques are too lengthy for our current analysis in a general cell biology laboratory. Gene sequencing has now been automated, and the key to success is the isolation of pure unique copy DNA (that is DNA that has only one sequence to analyze). Once sequenced, computer searches can be made of known libraries to compare the gene with that from other organisms.

For an excellent series of sequencing techniques cf. *Methods of DNA and RNA Sequencing*, ed. S.M. Weissman. Praeger Publishers, New York. 1983, or *Current Procols in MOlecular Biology* edited by F.M Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman and K.Struhl. Greene Publishing Associates, Wiley Interscience, Brooklyn, NY, 1988.

Further analyses of RNA are also possible. Affinity chromatography allows the separation of mRNA, which can then be analyzed for specific base sequence. This mRNA could be used for translation on isolated ribosomes, or used to synthetically produced DNA copies (cDNA).

Endnotes

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1. Modification of technique of Marmur (1961) as given in *Biochemical Methods in Cell Culture and Virology* by Robert J. Kuchler. Dowden, Hutchinson and Ross, Inc., Stroudsburg, 1977.
2. A scanning spectrophotometer may be used, if available.
3. The mathematics for this correction can be found in C. Mandel, M., and J. Marmur. 1968. "Use of ultraviolet absorbance temperature profile for determining the guanine plus cytosine content of DNA." In *Methods in Enzymology*, Vol. SII (L. Grossman and K. Moldave, eds.), pp. 195-206. Academic Press, Inc., New York.
4. Kirby and Parish, *Biochem J* 96,266. 1965.
5. Directions for for use of a Beckman SW27 rotor with a capacity of 34 ml. Volumes must be adjusted according to the capacity of the rotor employed.
6. *The Cell Nucleus* (H.Busch, ed.), pp 152-208. Academic Press, Inc. New York.
7. There are many ways to accomplish this, but the simplest is to use a descending strip of paper in a chromatography tank. This can be constructed from readily available pyrex houseware, glass rods and a bell jar, or by using commercial units. Alternatively, a simple ascending system can be made from large testtubes.

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Chapter 15: The Central Dogma

Introduction

The Central Dogma of modern biology is the conversion of the genetic message in DNA to a functional mRNA (transcription) and subsequent conversion of the copied genotype to a phenotype in the form of proteins.

The process of conversion of a mRNA to a functional protein is known as translation. It involves the attachment of a messenger RNA to the smaller subunit of a ribosome, the addition of the larger subunit, plus initiation by a host of other factors. The entire process can be accomplished in the absence of a cell, if all of the necessary factors are present. 1

Unfortunately, studies on translation and post-translational changes in protein structure are rather complex. They require a heavy investment in time and equipment. To some extent, the electrophoretic identification of proteins is part of this process, and the appearance of specific proteins can be monitored during any of the developmental processes identified in Chapter Thirteen. Chapter Fourteen lists several means of studying both the DNA and RNA contents of cells during changes.

To study the process of translation in any meaningful way requires that reasonably purified sources of mRNA, ribosomes and all of the amino acids be available. In addition, there is a requirement for various factors responsible for peptide chain initiation on the ribosome.

Consequently this chapter will deal with only one exercise. It is definitely an advanced technique, and requires mastery of many of the techniques presented in previous chapters. It must be performed on an independent basis, since the extensive time commitment does not lend itself to typical laboratory periods.

Exercise 15.1 - Protein Syntheses in Cell Free Systems

LEVEL III

Materials

- Suspension culture of fibroblast cells (1 liter)
- 35 mM Tris-HCl, pH 7.4, 140 mM NaCl (TBS buffer)
- 10 mM Tris-HCl, pH 7.5, 10 mM KCl, and 1.5 mM magnesium acetate (TBS-M)
- 10X TBS-M

  200 mM Tris-HCl, pH 7.5, 1200 mM KCl, 50 mM magnesium acetate and 70 mM β-mercaptoethanol

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- 10X solution of 20 amino acids
- Teflon homogenizer
- Refrigerated preparative centrifuge
- Sat. $(NH_4)_2SO_4$
- TBS-M plus 20% (v/v) glycerol
- 1X TBS-M buffer containing 0.25 M sucrose
- 1X TBS-M buffer containing 1.0 M sucrose
- Sephadex G-25 column equilibrated with 1X TBS-M buffer
- Liquid nitrogen storage
- Reaction mixture for protein synthesis, containing the following in a total volume of 50 µl [2]

Tris-HCl, pH 7.5	1.5 µm
Mg acetate	0.15 - 0.20 µm
KCL	4.0 - 5.0 µm
β-Mercaptoethanol	0.25 µm
ATP	0.05 µm
GTP	0.005 µm
Creatine phosphate	0.50 µm
Creatine kinase	8.0 µg
Each of 19 amino acids(-leucine)	2.0 nmol
^{14}C-leucine (150 mCi/mmol)	0.125 µCi
Ribosome fraction	1 to 2 A_{260} units
Viral mRNA or Globin 9S mRNA	2.0 to 5.0 µg
or	
Poly U[3]	10.0 µg

Procedure [4]

1. Chill the suspension culture (app. 10^9 cells) rapidly in an ice bath. Collect the cells as a pellet, by centrifugation at 600 xg for 10 minutes at 4° C. Resuspend the cells in TBS buffer and wash them three times with cold TBS buffer.
2. Suspend the final pellet in two volumes of TBS-M for 5 minutes at 0° C and homogenize the cells with 10 to 20 strokes in a tight fitting Teflon homogenizer.
3. For each 0.9 ml of homogenate, add 0.1 ml of concentrated 10X TBS-M buffer. Centrifuge the mixture at 10,000 xg for 10 minutes at 4° C.
4. Decant and collect the supernatant extract and adjust the extract such that the following are added to yield final concentration:

   ATP to 1.0 mM ATP
   GTP to 0.1 mM GTP
   Creatine phosphate to 10 mM
   Creatine kinase to 160 µg/ml
   Amino acids to 40 µm each

5. Incubate the mixture for 45 minutes at 37° C.

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6. Centrifuge the mixture at 10,000 xg for 10 minutes at room temperature. Cool the supernatant and pass the supernatant through a Sephadex G-25 column at 4° C.
7. Turn on a UV spectrophotometer and adjust the wavelength to 260 nm. Blank the instrument with TBS buffer.
8. Centrifuge the filtrate excluded from the Sephadex column at 165,000 xg for 90 minutes at 4° C.
9. Precipitate the proteins within the supernatant by the addition of saturated $(NH_4)_2SO_4$ to yield a final 60% $(NH_4)_2SO_4$. Collect the precipitate by centrifugation.
10. Dissolve the precipitate in TBS-M buffer and dialyze it against the same buffer containing glycerol.
11. Suspend the resulting ribosome pellet in 1X TBS-M buffer containing 0.25 M sucrose. Place 5 ml of TBS buffer with 1.0 M sucrose into the bottom of a centrifuge tube and layer the suspended ribosomes on top. Centrifuge at 216,000 xg for 2.5 hours at 4° C.
12. Wash the resulting pellet with TBS-M buffer, and resuspend it in the same buffer with 0.25 M sucrose.
13. Determine the ribosome concentration using a UV spectrophotometer to measure the A_{260}. The extinction coefficient for ribosomes is 12 A units per mg per ml at 260 nm.
14. The ribosomes may be frozen and stored in liquid nitrogen, or used for *in vitro* protein synthesis. If frozen, they should be thawed only once prior to use.

    To test for protein synthesis, prepare the reaction mixture for protein syntheis.

15. Incubate the reaction mixture at 37° C for 60 minutes. Terminate the reaction by pipetting 40 µl of the mixture onto a 2.5 cm disk of Whatman 3MM filter paper. Dip the disk into cold 10% TCA for 15 minutes and then in 5% TCA at 90° C for 15 minutes.
16. Rinse the disk twice in 5% TCA for 5 minutes, once in alcohol:ether (1:1), and then dry it.
17. Place the disk into a scintillation vial and add a toluene-based fluor. Refer to Appendix H for details on the use of a scintillation counter.
18. Measure the amount of radioactively labeled amino acid incorporated into protein.
19. Graph the protein synthesized versus time.

Optional

For advanced work, compare the activity of ribosomes isolated from the fibroblast cultures to those isolated from a prokaryote culture, a plant (yeast or pea seedlings) and from genetic µ tants known to alter the structure of either rRNA or any of the ribosome structural proteins.

If sources of mRNA are available (or if the time is taken to extract RNA and electrophoretically purify it), then comparisons of the rate of protein synthesis can be made within this system. Finally, once the proteins are synthesized, they can be separated on SDS-PAGE and rates of synthesis can be determined by both time of incorporation of leucine and by increasing molecular weights. The latter will also demonstrate the possible presence of µ ltiple RNA's with the system, whereas the radioactivity will mask the presence of heterogenous RNA.

Endnotes

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1. For an excellent complete laboratory procedure on the regulation of translation during the development of

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Tenebrio refer to: "Regulation of Messenger RNA Translation in Development: The critical Role of Transfer RNA" by Joseph Ilan. An Addison-We sley Module in Biology. No. 5. Addison-Wesley Publishing Company, Inc. 1973.

2. Falcoff, E., R. Falcoff, B. Lebleu, and M. Revel. 1973. Correlation between the antiviral effect of interferon treatment and the inhibition of in vitro mRNA translation in noninfected L cells. *J Virol*. 12:421-430.
3. When poly U is used, 0.125 µCi of ^{14}C-phenylalanine is used instead of the amino acids, and the magnesium acetate concentration is increase d to 0.25 µm.
4. Kuehler, Robert J. *Biochemical Methods in Cell Culture and Virology*. Dowden, Hutchinson & Ross, Inc. Sroudsburg, 1977. pp. 305-307.

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Appendix A: Units and Measures

SIZE

Cell biology deals with things which are relatively small. The units of measurement typically used are the micron at the light microscope level, and the nanometer at the electron microscope level. For molecular measurements, the norm is the Angstrom. These units are defined within the following table:

Measure	Symbol	Relative Length	Exponential Notation
Meter	M	1	10^0
Decimeter	dm	.1	10^{-1}
Centimeter	cm	.01	10^{-2}
Millimeter	mm	.001	10^{-3}
Micrometer or micron	µ	.000001	10^{-6}
Nanometer	nm	.000000001	10^{-9}
Angstrom	Å	.0000000001	10^{-10}

From this table it is apparent that:

10 Å = 1 nm
1000 nm = 1 mm
10 mm = 1 cm

Not apparent are that:

1 inch = 2.54 cm = 25.4 mm = 25,400 µ = 25,400,000 nm
1 inch = 2.54 cm = 2.54 X 10^1 mm = 2.54 X 10^4 µ = 2.54 X 10^7 nm
1 mm = 0.04 inches

VOLUME

Volumes are measured relative to a liter, with the most commonly used measurements, the milliliter and the microliter. The following table gives the relative volumes:

Measure	Symbol	Relative Volume	Exponential Notation
Liter	L	1	10^7
Deciliter	dl	.1	10^{-1}
Millimeter	ml	.001	10^{-3}
Microliter	µ l	.000001	10^{-6}

There are 1,000 µ l in 1 ml.

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1 ml = 1 cm³
1 gallon = 3.8 liters
1 quart = 0.95 liters
1 liquid ounce = 29.6 ml

WEIGHT

The most common measurements of weight at the gram, milligram and microgram.

Measure	Symbol	Relative Weight	Exponential Notation
Kilogram	Kg	1000	10^3
Gram	g	1	10^0
Milligram	mg	.0001	10^{-3}
Microgram	µ g	.0000001	10^{-6}

CONCENTRATION

Most concentrations used throughout cell biology are those of a solute disolved or suspended within a solvent, and in most cases the solvent is water. There are two general methods of identifying the concentration of a solution; as molarity or as a percent. Molarity is based on the number of moles of solute in the solvent, while percent is based on the number of parts , either grams (for a solid solute) or milliliters (for a liquid solute).

Molarity equals the number of moles of solute in 1 liter of solution. A mole is equal to the gram molecular weight (or formula weight) of the solute. Sodium Chloride (NaCl), for example has a formula weight of 58.43 (22.98 for Na and 35.43 for Cl). Thus, if 58.43 grams of NaCl are dissolved in 1 liter of water, the result would equal a 1 molar solution of NaCl. This is designated as 1 M NaCl, or as simple M NaCl.

We often deal with solutions of less volume than 1 liter, and the following should be noted:

1 M NaCl = 58.43 grams / liter = 58.43 mg/ml = 58.43 µ U g/µ U l.

A 0.002 M NaCl solution contains 0.002 moles of NaCl or 0.1168 grams (0.002 x 58.43) in one liter of solvent. Note that molar is abbreviated as M, but that there is no abbreviation for moles. The 0.002 M solution contains 0.002 moles (or 2 millimoles) or solute in one liter. A 0.002 M solution would contain 0.001 moles of solute in a half liter.

The number of moles = Volume (in liters) x Molar Concentration
The number of millimoles = Volume (in ml) x Molar Concentration

Note that chemical equations are always balanced via moles. Moreover, note that for dilutions of known concentrations, one can use the simple formula:

Molarity x Volume = Molarity x Volume

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If you have a 0.002 M solution of NaCl and you wish to obtain 100 ml. of a 0.001 M solution,

> 0.002 M x Needed Volume = 0.001 M x 100 ml
> Needed Volume = 0.001 M x .1 liters / 0.002 M = .050 liters= 50 ml.
> Measure 50 ml of the 0.002 M solution, and dilute it to 100 ml with the solvent (usually water, or an appropriate buffer).

Molarity is appropriate for use when chemical equations are to be balanced. When we deal with physical properties of solutions, molarity is not as valuable as a similar measurement of concentration, molality. For colligative properties of solutions (freezing point depression, boiling point elevation, osmotic pressure, density, viscosity), there is a better correlation between the property and molality.

Molality (designated with a lower case m) is equal to the number of moles of solute in 1000 gm of solvent. At first this may not appear any different from molarity, since a ml of water equals 1.0 gm. Indeed, for dilute solutions in water, there is little or no practical difference between a molar solution and a molal solution. In concentrated solutions, with temperature fluctuations and with changes in solvent, there is appreciable difference.

For example:

A 2 m (2 molal) solution of sucrose contains 684.4 gm of sucrose (twice the molecular weight or two moles of sucrose) dissolved in 1000 gm (approximately 1 liter) of water. The weight of this solution is 684.4 gm + 1000 gm or 1684.4 gm.

This solution (2 m sucrose) has a density of 1.18 gm/ml or 1180 gm/liter.

Since there are 1684.4 gms, division by the density (1180 gm/liter) would indicate that there are 1.43 liters of solution. That is, 684.4 gm of sucrose dissolved in 1000 gm of water would yield 1.43 liters of solution. This solution would contain 2 moles of sucrose, however and would have a molarity = 2 moles/1.43 liters or 1.40 M. So, a 2 m sucrose solution equals a 1.4 M sucrose solution.

PERCENT SOLUTIONS

In the example above of 2 m sucrose, there were 684.4 gm of sucrose in the final solution which weighed 1684.4 gm (684.4 gm sucrose + 1000 gm water). The percent of sucrose on the basis of weight is therefore 684.4/1684.4 x 100, or 40.6%.

There are three means of expressing concentration in the form of a percent figure:

1. Percent by weight (w/w); gm solute / 100 gm solvent
2. Percent weight by volume (w/v); gm solute /100 ml solvent
3. Percent by volume (v/v); ml solute / 100 ml solution

For dilute solutions, these differences are not significant, but at higher concentrations, they are. Chemists (when they use Percent designations) usually use w/w. Biochemists and physiologists

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more often use w/v. Both use v/v if the solute is a liquid. It is important to distinguish among these alternatives.

Using ethanol as an example, consider a 20% solution of ethanol in water, mixed according to the three designations of w/w, w/v and v/v.

1. w/w would contain 20 g of absolute ethanol mixed with 80 gm of water to yield a 20% (w/w) solution.
2. w/v would contain 20 g of absolute ethanol mixed with water to form a final volume of 100 ml.
3. v/v would contain 20 ml of absolute ethanol diluted to 100 ml with water.

The three solutions are not the same. First, the density of alcohol is not equal to that of water, and thus conversion of g to ml is not equivalent. A 20% (w/w) solution of ethanol, for example, has a density of 0.97 g/ml and 20 gm of ethanol plus 80 gm of water would have a volume of 103 ml. The % (w/v) for this solution would be 20 gm ethanol / 103 ml, or 19.4% (w/v). Similarly, absolute ethanol has a density of 0.79 gm/ml and thus 20 ml of ethanol would weigh 15.8 gm. A 20% (v/v) solution would contain 15.8 gm of ethanol in 100 ml and be a 15.8% (w/v) solution.

So, for ethanol:

20% (w/w) = 19.4 % (w/v)
20% (w/v) = 20.0 % (w/v)
20% (v/v) = 15.8 % (w/v)

In cell biology, the most common use of Percent solution is as (w/v). In practice, these are simple solutions to mix. For a 20% (w/v) sucrose solution, for example, simply weigh 20 gm of sucrose and dissolve to 100 ml with water.

Unless specifically stated otherwise, solutions lacking the appropriate designation should be assumed to be (w/v).

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Appendix B: Statistics

Statistical manipulation is often necessary to order, define and/or organize raw data. A full analysis of statistics is beyond the scope of this work, but there are some standard analyses that anyone working in a cell biology laboratory should be aware of, and know how to perform. After data is collected, it must be ordered, or grouped according to the information which is to be sought. Data is collected in the forms:

Type of Data	Type of Entry
Nominal	yes or no
Ordinate	+, ++, +++
Numerical	0, 1, 1.3, etc.

When collected, the data may appear to be a mere collection of numbers, with little apparent trends. It is first necessary to order those numbers. One method is to count the times a number falls within a range increment. For example, in tossing a coin, one would count the number of Heads and Tails (eliminating the possibility of it landing on its edge). Coin flipping is nominal data, and thus would only have two alternatives. Should we flip the coin 100 times, we could count the number of times it lands Heads and the number of Tails. We would thus accumulate data relative to the categories available. A simple table of the grouping would be known as a frequency distribution , for example:

Coin Face	Frequency
Heads	45
Tails	55
Total	100

Similarly, if we examine the following numbers; 3,5,4,2,5,6,2,4,4, several things are apparent. First, the data needs to be grouped and the first task is to establish an increment for the categories. Let us group the data according to integers, with no rounding of decimals. We can construct a table which groups the data.

Integer	Frequency	Total (Integer x Frequency)
1	0	0
2	2	4

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3	1	3
4	3	12
5	2	10
6	1	6
Totals	9	31

MEAN, MEDIAN AND MODE

From the data, we can now define and compute three important parameters of statistics.

Definition

The mean: The average of all the values obtained. It is computed by the sum of all of the values (Σ x), divided by the number of values (n). The sum of all numbers is 31, while there are 9 values, thus, the mean is 3.44.

$$\bar{M} = \Sigma\ x / n$$

Definition

The median: The mid point in an arrangement of the categories by magnitude. Thus, the low for our data is 2, while the high is 6. The middle of this range is 4. The median is 4. It represents the middle of the possible range of categories.

Definition

The mode: The category that occurs with the highest frequency. For our data, the mode is equal to 4, since it occurs more often than any other value.

These values can now be used to characterize distribution patterns of data.

For our coin flipping, the likelihood of a Head or a Tail is equal. Another way of saying this is that there is equal probability of obtaining a Head or not obtaining a Head with each flip of the coin. When the situation exists that there is equal probability for an event as for the opposite event, the data will be graphed as a binomial distrution, and a Normal curve will result. If the coin is flipped ten times, the probability of one head and nine tails equals the probability of nine heads and one tail. The probability of two heads and eight tails equals the probability of eight heads and two tails and so on. However, the probability of the latter (two heads) is greater than the probability of the former (one head). The most likely arrangement is five heads and five tails.

When random data is arranged and displays a binomial distribution, a plot of frequency vs. occurency will result in a normal distribution curve . For an ideal set of data (i.e. no tricks, such as two headed coin, or gum on the edge of the coin), the data will be distributed in a bell shaped curve, where the median, mode and mean are equal.

This does not give an accurate indication of the deviation of the data, and in particular does not inform us of the degree of dispersion of the data about the mean. The measure of the dispersion of data is known as the Standard Deviation . It is given mathematically by the formula:

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$$S = \mathrm{sqrt}(\Sigma(M - \bar{X})^2 / n-1)$$

This value gives a measure of the variability of the data, and in particular, how it varies from an ideal set of data generated by a random binomial distribution. In other words, how different is it from an ideal Normal Distribution. The more variable the data, the higher the value of the standard deviation.

Other measures of variability are the range (difference between minimum and maximum values), the Coefficient of Variation (Standard Deviation divided by the Mean and expressed as a Percent) and the Variance.

The variance is the deviation of several or all values from the mean and must be calculated relative to the total number of values. Variance can be calculated from the formula:

$$V = \Sigma(M - \bar{X})^2 / n - 1$$

All of these calculated parameters are for a single set of data that conforms to a normal distribution. Unfortunately, biological data does not always conform in this way, and often sets of data must be compared. If the data does not fit a binomial distribution, often it fits a skewed plot known as a Poisson distribution . This distribution occurs when the probability of an event is so low, that the probability of its not occurring approaches 1. While this is a signficant statistical event in biology, details of the Poisson Distribution are left to texts on biological statistics.

Likewise, the proper handling of comparisons of multiple sets of data. Suffice it indicate that all statistics comparing multiple sets begin with calculation of the parameters detailed here, and for each set of data. For example, the standard error of the mean (also known simply as the standard error) is often used to measure distinctions among populations. It is defined as the standard deviation of a distribution of means. Thus, the mean for each population is computed and the collection of means are then used to calculate a standard deviation of those means.

Once all of these parameters are calculated, the general aim of statistical analysis is to estimate the significance of the data, and in particular the probability that the data represents effects of experimental treatment, or conversely, pure random distribution. Tests of significance (Student's t Test, Analysis of Variance and Confidence Limits) will also be left to more extensive treatment in other volumes.

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Appendix C: Graphs

Presentation of data in an orderly manner often calls for a graphic display. This is made somewhat easier today, with the advent of graphics programs for the personal computer, but still requires the application of some basic techniques.

The first consideration for a graph, is whether the graph is needed, and if so, the type of graph to be used. For accuracy, a well constructed table of data usually gives more information than a graph. The values obtained and their variability are readily apparent in a table, and interpolation (reading the graph) is unnecessary. For visual impact, however, nothing is better than a graphic display.

There are a variety of graph types to be chosen from; e.g. line graphs, bar graphs and pie graphs. Each of these has its own characteristics and subdivisions. One also has to decide upon singular or multiple graphs, two-dimensional or three dimensional displays, presence or absence of error bars, and the aesthetics of the display. The latter include such details as legend bars, axes labels, titles and selection of the symbols to represent data, and patterns for bar graphs.

Selection of a good Graphic Program for computer use will make most of these choices available. Refer to Appendix D for a quick review of such programs.

THE BASICS:

Perhaps the number one rule for graphic display has to do with the axes. Given a two-dimensional graph, with two values (x and y), which value is x and which is y? The answer is always the same - the KNOWN value is always the ordinate (x) value. The value that is MEASURED is the abscissa (y) value. For a standard curve of absorption in spectrophotometry, the known concentrations of the standards are placed on the x axis, while the measured absorbance would be on the y. For measurements of the diameter of cells, the x axis would be a micron scale, while the y axis would be the number of cells having a given diameter.

Unless you are specifically attempting to demonstrate an inverted function (which is confusing at best), the scales should always be arranged with the lowest value on the left of the x axis, and the lowest value at the bottom of the y value. The range of each scale should be determined by the lowest and higest value of your data, with the scale rounded to the nearest tenth, hundredth, thousandth, etc. That is, if the data ranges from 12 to 93, the scale should be from 10 to 100. It is not necessary to always range from 0, unless you wish to demonstrate the relationship of the data to this value (as for example in a Lineweaver-Burke plot of enzyme kinetics, or a Spectrophotometric standard curve).

The number of integrals placed on the graph will be determined by the point you wish to make, but in general, one should use about ten divisions of the scale. For our range of 12 to 93, an appropriate scale would be from 0 to 100, with an integral of 10. Placing smaller integrals on the scale does not convey more information, but merely adds a lot of confusing marks to the graph.

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The user can estimate the values of 12 and 93 from such a scale without having every possible value ticked off on a scale.

An important rule of scale deals with multiple graphs drawn separately. If the graphs are to be compared, the scales MUST REMAIN CONSTANT. Nothing is more disconcerting than to be shown two graphs with varying axis values and being asked to compare the two. It would be better to merely tabulate the data than to graphically present it.

LINE GRAPH VS. BAR GRAPH OR PIE GRAPH

If the presentation is to highlight various data as a percent of the total data, then a pie graph is ideal. Pie graphs might be used for example to demonstrate the composition of the white cell differential count. They are the most often used graph type for business use, particularly in displaying budget details.

Pie Graphs are circular presentations which are drawn by summing your data and computing the percent of the total for each data entry. These percent values are then converted to portions of a circle (by multiplying the percent by 360 °) and drawing the appropriate arc of a circle to represent the percent. By connecting the arc to the center point of the circle, the pie is divided into wedges, the size of which demonstrate the relative size of the data to the total. If one or more wedges are to be highlighted, that wedge can be drawn slightly out of the perimeter of the circle for what is referred to as an exploded view.

More typical of data presented in cell biology, however, are the line graph and the bar graph. There is no hard and fast rule for choosing between these graph types, except where the data is non-continous. Then, a bar graph must be used. In general, line graphs are used to demonstrate data which is related on a continous scale, whereas bar graphs are used to demonstrate discontinous or interval data.

Suppose, for example, that you decide to count the number of T-lymphocytes in four slices of tissue, one each from the thymus, Payer's patches, a lymph node and a healing wound on the skin. Let's label each of these as T, P, L and S respectively. The numbers obtained per cubic centimeter of each tissue are T=200, P=150, L=100 and S=50. Note that there is a rather nice linear decrease in the numbers if T is placed on the left of an x axis, and S to the right. A linear graph of this data would give a nice straight line, with a statistical regression fit and slope. But look at the data! There is no reason to place T (or P,L or S) to the right or left of any other point on the graph - the placement is totally arbitrary. A line graph for this data would be completely misleading since it would imply that there is a linear decrease from the thymus to a skin injury AND that there was some sort of quantitative relationship among the tissues. There is certainly a decrease, and a bar graph could demonstrate that fact, by arranging the tissue type on the x axis in such a way to demonstrate that relationship - but there is no inherent quantitative relationship between the tissue types which would force one and only one graphic display. Certainly, the thymus is not four times some value of skin (although the numbers are).!

However, were you to plot the number of lymphocytes with increasing distance from the point of a wound in the skin, an entirely different presentation would be called for. Distance is a continous variable. We may choose to collect the data in 1 mm intervals, or 1 cm. The range is continous from 0 to the limit of our measurements. That is we may wish to measure the value at 1 mm, or 1.2

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mm or 1.23 mm or 1.23445 mm. The important point is that the 2 mm position is 2x the point at 1 mm. There is a linear relationship between the values to be placed on the x axis. Therefore a linear graph would be appropriate, with the dots connected by a single line. If we choose to ignore the 1.2 and 1.23 and round these down to a value of 1, then a bar graph would be more appropriate. This latter technique (dividing the data in appropriate intervals and plotting as a bar graph) is known as a Histogram. This graph is very familiar to students since it is the graph used for the display of grade distributions.

Having decided that the data has been collected as a continous series, and that the data will be plotted on a linear graph, there are still decisions to be made. Should the data be placed on the graph as individual points with no lines connecting them (a Scattergram)? Should a line be drawn between the points (known as a Dot-to-Dot)? Should the points be plotted, but curve smoothing be applied? If the latter, what type of smoothing?

There are many algorhythms for curve fitting, with the two most commonly used being linear regression and polynomial regression. It is important to decide BEFORE graphing the data, which of these is appropriate.

Linear regression is used when there is good reason to suspect a linear relationship within the data (as for example in a spectrophotometric standard following the Beer-Lambert law). In general, the y value can be calculated from the equation for a straight line, $y = mx + b$, where m is the slope and b is the y- intercept.

Computer programs for this can be very misleading. Any set of data can be entered into a program to calculate and plot linear regression. It is important that there be a valid reason for supposing linearity before using this function, however. This is also true when using polynomial regressions. This type of regression calculates an ideal curve based on quadratic equations with increasing exponential values, that is $y = (mx+b)^n$, where n is greater than 1. The mathematics of this can become quite complex, but often the graphic displays begin to look better to the beginning student. It is important to note that use of polynomial regression must be warranted by the relationship within the data, not by the individual drawing the graph.

For single sets of data, that is the extent of the available options. For multiple sets the options increase. If the multiple sets are data collected pertaining to identical ordinate values, then error bars (standard deviation or standard error of the means) can be added to the graphics. Plots can be made where two lines are drawn, connecting the highest y values for each x, and a second connecting the lowest values (the Hi-Lo Graph). The area between the two lines presents a graphic depiction of variability at each ordinate value.

If the data collected involves two or more sets of data having a common x axis, but varying y axes (or values), then a multiple graph may be used. The rules for graphing apply to each set of data, with the following provision; Keep the number of data sets on any single graph to an absolute minimum. It is far better to have three graphs, each with 3 lines (or bars) than to have a single graph with 9 lines. A graph that contains an excess of information (such as 9 lines) is usually ignored by the viewer (as are tables with extensive lists of data). For this same reason, all

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unnecessary clutter should be removed from the graph; e.g. grid marks on the graph are rarely useful.

Finally, it is possible to plot two variables, y and z, against a common value, x. This is done with a 3D graphic program. The rules for designing a graph follow for this type of graph, and the use of these should clearly be left to computer graphics program. These graphs often look appealing with their hills and valleys, but rarely impart any more information than two separate 2D graphs. Perhaps the main reason is that people are familiar with two dimensional graphics, but have a more difficult time visually interpreting three dimensional graphs.

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Appendix D: Computers

The advent of the personal computer has been one of the most significant factors impacting laboratory work in the past decades. These tools are now readily available and can be used at every level of work. In the 1960's computer use was limited to larger institutions and invariably centered on the use of large main frame computers, primarily for data analysis. Their use was restricted to those with the willingness to learn complex high level languages, and the time and energies to write their own programs. Computers were somewhat unfriendly and the author can recall the most dreaded, yet common lament The computer is down, again! Central main frames and their later mini computer counterparts all too often went down in the midst of your work. The adage to save and save often was learned very quickly.

Although there were other pioneers, it was the introduction of the Radio Shack TRS-80 and the Apple computer systems which began a revolution. Those systems were closely followed by the introduction of the Commodore PET, and Kaypro computers. These early machines were marvels for their time, but were generally lacking in random access memory (RAM) and speed. Manufacturers also attempted to corner markets by intentionally designing hardware and software that was incompatable with other manufacturers (such as the lack of Commodore's use of ASCII [American Standard Code for Information Interchange] in its PET system and the substitution of its own standard, affectionately known among users as the Half-ASCII). Much of this changed in 1980 with the introduction of the IBM-PC. This machine, backed by the industry giant, set the standard that is still in vogue. Apple was well entrenched in academic circles by this time and has not given in to the pressure for an IBM standard. Apple responded with the McIntosh line, and the battle lines have been drawn since.

Which is a way of introducing the first decision that one faces when considering the use of a personal computer in cell biology. Which system should one invest in? For many institutions, this is not a consideration since one or another system has already been purchased by the institition. For others it remains, because either there is no computer available or there are many types available within the institution.

Let me settle the author's prejudice up front. It doesn't make any difference which hardware system you use. The important point is that the computer is used as a tool to enhance learning in the laboratory. An installed Radio Shack TRS-80 can be as useful as a new McIntosh II, Compaq 386/33, or a NeXT. To those still needing to acquire a computer system, talk with the institutional academic computing center and follow their advice. If you do not have such a center, contact one at the nearest university and follow their advice.

Of course availability of software programs should be considered, but in general, you can find what you need. Newer, faster and bigger machines make some projects more pleasant (fun?) but are rarely necessary. For most undergraduate instructional use of computers in the laboratory, any basic personal computer should suffice. It is a rare event in the cell biology laboratory where multi-tasking (having more than one program running simultaneously), networking (connection to

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other computers), or extensive real-time data analysis is required. In many instances, these activities actually detract from the student's ability to grasp the principle of the exercise. The computer may act like the Siren's of the Odyssey (which you may recall ended in a shipwreck).

Having said this, it is now appropriate to indicate that most of the hardware and software discussed in this section is that appropriate to the IBM standard (generally referred to as IBM compatable, and running under PC-DOS or its counterpart, MS- DOS). This is purely due to the use of this standard at the author's institution and is not meant as an endorsement of this standard over any other (such as Apple).

LANGUAGES Before discussing commercial programs, a brief note should be inserted about programming a computer. Better than 90% of all the uses of a computer in lab can be performed using commercially available programs. For many this number can easily approach the 100% mark. In other words, you may never have to actually write your own program.

However, as often happens, you may become quite dissatisfied with the program established by another and wish to modify it to your own purpose. It is at this point in time that you will need to write a program.

The list of available computer languages for writing such a program is as long as the list of computers. Everyone has their favorite, and nothing can provoke a heated discussion among computer users faster than the merits (or demerits) of any given program language. Computer hackers love to code programs in what is known as assembly language. This is nearly an unreadable code that takes immediate command of the machine and is the closest to machine code , the only code which the computer actually uses. Commercial programs are usually written in assembly code or something close, in order to increase performance speed. It is rarely used by cell biology laboratories.

All beginners start with a language which needs to be interpreted before assembly and conversion into machine language. Since all of this must take place, these languages tend to slow down performance, unless compiled. Unquestionably, the most common language used is some version of BASIC (Beginners All- purpose Symbolic Instructional Code). There are several reasons for this. First, it is usually bundled with the equipment (the hardware) and thus is readily available. Second, it follows a straight-forward algebraic logic in its construction. It has simple commands which are almost intuitive in their use. And finally, while it is not required that programs be unstructured, it is probably the most forgiving language available for sloppy programming.

Newer enhanced versions of BASIC (True-Basic, Turbo Basic or Quick Basic 4.5) have taken this language and elevated it to a point where it can compete with virtually any language for speed and flexibility. BASIC has the distinct advantage of being the most portable language between differing systems. The author's favorite is Microsoft's QuickBasic.

For other reasons, Math and Computer Departments have taken to instructing PASCAL, FORTRAN, FORTH, LISP, or C, to mention only a few. These are said to be more structured. Be wary, however. It is not necessary to write unstructured code in BASIC, and code that is written with these other languages, may not transfer easily from one machine to another, without

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extensive reworking. In practice, once again, it hardly matters in an undergraduate cell biology laboratory. For programming your own code, use the language you are most familiar with.

Programs given in this manual are in the more familiar BASIC format (IBM BASICA). Since even the core BASIC instructions vary slightly between computer systems, some conversion may be necessary prior to their use.

COMMERCIAL SOFTWARE There are a number of types of programs available and useful to the cell biology laboratory

- Spread Sheets
- Graph Programs
- Equation Solvers
- Data Base Programs
- Word Processors
- Outline Programs
- Paint/Draw Programs
- Computer Assisted Instruction (CAI)
- Single Purpose Programs

SPREAD SHEETS

Without question, the single most useful program available for data collection and analysis is the spread sheet program. There are several available, each with its own merits. The leader (in sales and business use) in the PC dominated field is Lotus 1- 2-3. Others of equal or superior value are Supercalc5, Quattro, or Excel. Integrated packages (Open Access, Symphony, Enable, Framework) nearly always contain powerful spreadsheets. Several of these offer educational discounts of their purchase price.

A spreadsheet program is an electronic balance sheet divided in rows and columns. Pioneered by Visicalc, these programs may have had as much impact on computer use as the actual design of the hardware. Any data that can be tabulated in columns and rows can be added to this type of program. Functions are readily available for totals (sums), averages, means, maximum and minimum values, and full trigonmetric functions. The programs listed above also include capabilities of sorting data, searching through the data, and for automatic graphing of the data. Each allows the construction of blank masks that contain the instructions for coding input and output while allowing students ease of data entry.

Spreadsheet programs have become so powerful in their latest versions, that they can be used as word processors and for data base manipulations, although they are not very sophisticated in these functions. Many of their graph routines are as powerful as stand alone programs for graphing, and are usually easier to use. The data entered can readily be transferred between other programs, from BASIC through direct analog conversions of integrated equipment.

If you were to purchase only one software package for the cell biology laboratory, it should be a spreadsheet.

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The use of a spread sheet can be demonstrated with the calculation of oxygen uptake by isolated mitochondria (Exercise 8.7). The data are collected for fourteen readings, every 10 minutes and must then be corrected for temperature and barometric fluctuations, as well as residual activity within the isolated mitochondria. The corrected data is then graphed. Use of a computer spreadsheet in the laboratory makes this a simple task, and allows visualization of the progress of the exercise as it is happening.

SuperCalc 4 listing of data from mitochondrial respiration

| A || B || C || D || E || F || G || H || I || J || K || L || M || N | O |

1 Mitochondrial Respiration

2 Cell Biology Exercise 8.7

3

4 Flask Number

5---

6 1 2 3 4 5 6 7 8 9 10 11 12 13 14

7 Time Manometer Readings

8---

9 10 506 500 490 490 445 499 496 503 585 497 499 510 493 493

10 20 510 490 487 487 416 492 493 512 490 504 501 516 500 500

11 30 522 489 479 479 394 478 490 511 490 506 505 518 497 497

12 40 536 486 476 476 370 475 501 525 499 513 513 527 515 515

13 50 555 486 473 473 345 458 505 526 499 514 515 527 515 515

14 60 575 485 469 469 327 456 508 526 502 516 517 529 513 513

15 70 590 482 464 464 305 448 510 529 506 525 524 532 518 518

16

17 Minutes Microliters Oxygen Consumed

18 Oxygen Utilization by Mitochondria

19

20

21 Change in gas volume (Average of replicates)

22 Flask Numbers (in pairs

23 Time 1/2 3/4 5/6 7/8 9/10 11/12 13/14

24 ---

25 10 -3.0 10.0 28.0 .5 -41.0 -4.5 7.0

26 20 .0 13.0 46.0 -2.5 3.0 -8.5 .0

27 30 -5.5 21.0 64.0 -.5 2.0 -11.5 3.0

28 40 -11.0 24.0 77.5 -13.0 -6.0 -20.0 -15.0

29 50 -20.5 27.0 98.5 -15.5 -6.5 -21.0 -15.0

30 60 -30.0 31.0 108.5 -17.0 -9.0 -23.0 -13.0

31 70 -36.0 36.0 123.5 -19.5 -15.5 -28.0 -18.0

32 Paired readings, averaged, and subtracted from original

33 Values corrected for Temp/Pressure/Endogenous

34---

35 .5 Glu.8 GluAzide DNP MalonaControl

36 Corrected for background

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37		3/4	5/6	7/8	9/10	11/12
38	10	6.0	24.0	-3.5	-45.0	-8.5
39	20	13.0	46.0	-2.5	3.0	-8.5
40	30	23.5	66.5	2.0	4.5	-9.0
41	40	50.0	103.5	13.0	20.0	6.0
42	50	62.5	134.0	20.0	29.0	14.5
43	60	74.0	151.5	26.0	34.0	20.0
44	70	90.0	177.5	34.5	38.5	26.0

Corrected values used to plot Figure D.1

GRAPHICS PROGRAMS

Separate programs for graphing data are useful in that they contain more options than those found within spreadsheets, and allow for more complex graphs. They are also designed for direct presentation of results and are capable of more polish. That is, there are selections of such things as fonts, error bars and statistical analyses and three dimensional presentations. The better programs will also give regressional analyses, either linear or polynomial. Nearly all allow for data input from a spreadsheet or data base manager, in addition to direct entry.

Among the best in this area, are Sigma Plot, Energraphics, Harvard Graphics and Cricket Graph. Most of these programs (except Sigma Plot) are designed for business graphics, but can be used in the cell laboratory.

EQUATION SOLVERS

In this category, there is not a lot of choice of programs. There are only two that come readily to the fore, TK-Solver and Borland's Eureka.

These programs allow for rapid entry of data for variables and simultaneous calculation of any other parameter within an equation. TK-Solver is ideal for insertion of such things as the Nernst equation, or the Hodgkin-Huxley equation, with subsequent query of What-if questions. If you wish to attempt mathematical modeling of cAMP pulsing in D. *discoideum* , TK-Solver or Eureka are well worth the investment.

DATA BASE MANAGERS

These programs are powerful for the long term storage and manipulation of data. They are more useful for research storage of data than for direct use in the undergraduate laboratory. Their strength lies in the ability to do with words (alphanumeric) what spreadsheets can do with numbers.

The leader in the business field is DB II (III and IV), but there are equal programs available for a lower cost. Among those to be considered are Q&A, Reflex, R-base and PC File. For ease of use, while retaining powerful options of data manipulation, Q&A excells. For tabular presentations of data, Reflex is the choice. For relational needs, R-base or the DB series are the programs of choice. PC File represents a good, less expensive alternative.

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Data base managers are excellent means of filing references, sources and such things as equipment lists, chemical inventory, etc. They can also be used effectively for filing of nucleic acid sequences, but the data base must be created before it can be used. Whether or not to use a data base manager for this purpose depends on the availability of the data in an appropriate form for use within your program. If data base managers are used, however, a hard drive becomes nearly mandatory.

WORD PROCESSORS

There are perhaps as many word processors available as pebbles on the shore. For many computer users, this function is synonymous with computers. For purposes of the cell biology laboratory, any one is sufficient. The programs are useful for writing lab reports, and marginally useful as alternatives to data base managers for such things as searching for nucleic acid codes.

Virtually all word processors have a Search function, and this function can be used to search a code several thousand letters long for the presence say of a 10 base code. This is not recommended, however, since the process is usually slow from within a word processor, and most word processing programs have severe limitations on the length of the words searched.

Several word processor packages are available for scientific presentation, and these will make inclusion of scientific formulas and symbols easier.

Several are equipped with the ability to perform red- lining and can compare two files for any changes. This is useful if you ask for rewriting of lab reports. The computer will highlight those portions of the report which have been changed in the second version, eliminating the need to review the other sections. This feature is also useful for group reports, where several individuals may make changes in the presentation. By far the most popular word processor is Word Perfect. The author's personal choice is XyWrite III, which was used for this publication.

Closely allied with word processors are Desk Top Publishers . These programs can format both text and graphics for typesetter output, or for printing on laser printers. These programs are rarely directly useful in the undergraduate lab, but are excellent when it comes time to communicate your information to others. The use of Xerox Ventura Publisher or Aldus Pagemaker (the two representing the top of the line) is left for final publication layout. Ventura Publisher, for example, was used for the final draft of this manual.

Desk Top Publishers are mentioned in this section merely as an option. Many software updates of familiar word processors are including the capabilities of desk top publishing into their programs. For the most part, this adds needless expense to the update, and increases the learning curve significantly. More importantly, it increases the need for larger quantities of RAM and faster hardware. This adds to the cost of the system and although it may add impressive screen graphics, it rarely adds to any improved laboratory outcome.

There is a good deal of hype circling around which word processor is the best . The answer is simple. If you have a word processor, you know how to use it, and it works for you, keep it.

OUTLINE PROGRAMS

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These programs are also integrated within some word processors, or can be purchased separately. The separate packages are primarily MaxThink or ThinkTank. These programs are an excellent means for groups or individuals to organize a presentation (lab report, seminar) or even to design a protocol. The programs allow the user to wander over the possibilities for presentation, and the program takes over the organization of those thoughts into an outline form (not without some human intervention, of course).

The author has found these programs to be of value to students in formulating the purpose of individual laboratory exercises, when linked to either reports or individual projects. The entire outline for this manual was first conceived and organized using MaxThink.

If you are heavily into writing reports and/or student papers, you may want to tie together a good outlining program with a wordprocessor and top it off with RightWriter. The latter is a grammar and context checker that is quite enlightening when applied to literary creations.

PAINT / DRAW PROGRAMS

A useful adjunct to various graph programs are those listed as paint or draw . Paint programs are those which plot pixels on a graphic screen and allow simple drawing routines. Representative of these are PC-Paintbrush, PC-Paint, and PaintShow. The latter was used for most of the simple computer graphics in this manual. Draw programs use mathematical formulas for their graphics (as opposed to pixel maps). They give excellent resolution, but generally are more expensive (the top of the line AutoCad is over $2,000), and require significantly more planning than the paint programs. If you need good graphics, however, use a draw program. McIntosh simply excels in this area. If integrated graphics are to be used extensively, the McIntosh is without question the superior environment. On the IBM side, any of the programs running under Microsoft's GEM Draw are good, and a batch of new programs running in the Microsoft Windows environment are approaching the quality and ease of the McIntosh formula. The easiest to use (learn) is perhaps Corel Draw!. Much of the line art for this manual was scanned or drawn using this program, with subsequent printing by an HP IIP laser printer at 300 DPI.

CAI

Computer Aided Instruction is a large field, and the best advice is let the buyer beware . There are some excellent programs available for instruction. Most are rather simplistic in their approach, graphics and design. Many are nothing more than childish diagrams of organs and cells which could be better presented with a real photomicrograph (and not tie the student to the monitor). At their worst, these programs become mere page flippers where instead of turning the page on a book, the computer regenerates a new screen crammed with information to be read. Their only value is for the student (now rare) who is awed by the computer and thus might turn the page.

At their best, these programs can be completely interactive and can readily and accurately simulate laboratory conditions that are time or cost prohibitive. An excellent example of this is the use of computer simulation for enzyme kinetics.

These programs are so numerous and variable, that use of them is left to the instructors. The best means of examining them is attendance at national meetings where they can often be reviewed. There are also user's groups which will review programs. The only means of deciding on the

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purchase and use of CAI programs is to talk with those who have used them (not the people who wrote them or are selling them) and then try them yourself.

SINGLE PURPOSE PROGRAMS.

These programs are sometimes available through commercial channels, but more often are written for specific purposes. It would be impossible to list all of the areas where these could be used in a cell biology laboratory, but a few should be mentioned.

- Resolution Calculations for Light and EM Microscopy
- Cell Morphometry - Area, Volume, Numbers
- Centrifugation, Conversion among rotors
- Centrifugation, Sedimentation Coefficients, Molecular Weights
- Beer-Lambert law
- Calculation of Molecular Weights from Electrophoresis Gels
- Ion Flux across Membranes
- Manometric Calculations
- H^+ flux and Chemiosmosis
- Gene Mapping, Recombination
- Growth Curves
- Simulation of cAMP effects on Dictyostelium
- Radiation Dose Response

Essentially, anything which requires repetitive calculations or data sorting is material for this section.

Included in this appendix are two programs. The first (CELLM) allows the student to play the role of a cell membrane. The second, (BEER) allows rapid calculations of extinction coefficients using the Beer-Lambert law. Other programs are available from the author, but space prohibits extensive listings. For example, a QuickBasic program for interconversion of centrifuge rotors and calculations of viscosity, sedimentation coefficients and clearing constants would require 12-15 pages if typed out. Programs can become very complex over time. An example of a page flipper program (for review of photosynthesis) originally written in PC Basic and converted to QuickBasic 4.5 is longer yet. This type of program can be fun, since graphics can be included to demonstrate chlorophyll accepting light (as lightning bolts) and transferring electrons to other molecular orbits. There are several commercially available programs of this type.

CELLM program (Membrane control of metabolites) 1

```
10 REM BY KEN WITT      TRACY HIGH SCHOOL
20 REM MODIFIED FOR BASIC-V    13 JUNE 1979        K. ANDERSON
30 REM MODIFIED FOR IBM    BY SCOTT SHEKELS          MARCH 3, 1986
40 REM                Gustavus Adolphus College
50 REM ***************************************************
60 REM
70 REM
80 READ H,S,K,N,C,W,P
```

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```
90 DATA 6700,30,53,0,1,0,0
100 WIDTH 40:COLOR 15,1,1:KEY OFF:CLS
110 LOCATE 1,15:PRINT"WELCOME!"
120 LOCATE 5,18:PRINT"TO"
130 LOCATE 9,16:PRINT"CELLM"
140 LOCATE 11,6:PRINT"A CELL MEMBRANE SIMULATION."
150 FOR SCOTT=1 TO 2000:NEXT:CLS
160 PRINT    "Do you wish to see an explanation"
170 PRINT    "of this program";
180 INPUT A$:A$=LEFT$(A$,1)
190 IF A$="Y" OR A$="y" THEN 210
200 GOTO 720
210 WIDTH 80:COLOR 15,1,1:CLS
211 PRINT     "IN THIS PROGRAM YOU WILL BE ROLE PLAYING. YOU WILL ACT AS"
220 PRINT     "A CELL MEMBRANE AND YOUR RESPONSIBILITY WILL BE TO MAKE"
230 PRINT     "ADJUSTMENTS IN THE FOLLOWING ITEMS:"
240 PRINT     "1.WATER"
250 PRINT     "2.SUGAR"
260 PRINT     "3.POTASSIUM IONS"
270 PRINT     "4.SODIUM IONS"
280 PRINT     "5.CHLORIDE IONS"
290 PRINT     "6.WASTE(PRIMARILY AMMONIA COMPOUNDS)"
300 PRINT     "-----------------------------------------------"
310 PRINT     "SAFE CONCENTRATIONS ARE AS FOLLOWS:"
320 PRINT     "               WATER BETWEEN 6000 AND 8000 MOLECULES"
330 PRINT     "               SUGAR BETWEEN 10 AND 35 MOLECULES"
340 PRINT     "               POTASSIUM BETWEEN 40 AND 53 IONS"
350 PRINT     "               SODIUM BETWEEN 0 AND 13 IONS"
360 PRINT     "               CHLORIDE BETWEEN 1 AND 3 IONS"
370 PRINT     "               WASTE BETWEEN 0 AND 10 MOLECULES"
380 PRINT"PRESS RETURN TO CONTINUE"
390 A$=INKEY$:IF A$="" THEN 390
400 CLS
410 PRINT     " "
420 PRINT     " "
430 PRINT     "YOU WILL BE A CELL MEMBRANE OF ESCHERICHIA COLI. YOUR"
440 PRINT     "CONCENTRATIONS ARE:"
450 PRINT     "               WATER-67% OR 6700 MOLECULES         SUGAR-30 MOLECULES"
460 PRINT     "               POTASSIUM IONS-53                   NO SODIUM OR WASTE"
470 PRINT     "               A TRACE OF CHLORIDE ION"
480 GOTO 1900
490 CLS:PRINT      " "
500 PRINT     "-------------------------------------------------------"
510 PRINT     "UNDERSTAND,    AS YOU DO ANY OF THE FOLLOWING, ENERGY WILL"
520 PRINT     "BE USED UP IN THE AMOUNTS SHOWN."
530 PRINT     "          GET RID OF WASTE-4 MOLECULES OF SUGAR"
```

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```
540 PRINT     "-------------------------------------------------------"
550 PRINT     "CHANGES IN THE CELL DUE TO OSMOSIS AND DIFFUSION TAKE"
560 PRINT     "PLACE AUTOMATICALLY. OTHER ENERGY FOR OTHER FUNCTIONS"
570 PRINT     "OF THE CELL, SUCH AS PREPARATION FOR REPRODUCTION ALSO"
580 PRINT     "DEPRICIATE AUTOMATICALLY."
590 PRINT     "WASTE BUILD UP FROM SUCH ENERGY ALSO TAKES PLACE."
600 PRINT     " "
610 PRINT     "PRETEND YOU ARE NOW PLACED INTO A SOLUTION WHICH HAS THE"
620 PRINT     "FOLLOWING CONSTANT CONCENTRATIONS:"
630 PRINT     "          WATER-87%                SUGAR-10%              SODIUM-TRACE"
640 PRINT     "          POTASSIUM-TRACE          CHLORIDE-TRACE"
650 PRINT     "*********************************************************"
660 PRINT     "THE CELL    BEGINS TO BURN SUGAR FOR ENERGY. DIFUSION AND"
670 PRINT     "OSMOTIC POTENTIALS GO INTO EFFECT. YOU ARE NOW READY TO"
680 PRINT     "ATTEMPT TO KEEP THIS CELL ALIVE!"
690 PRINT     "================================="
700 PRINT:PRINT"PRESS RETURN TO CONTINUE";
710 A$=INKEY$:IF A$="" THEN 710
711 WIDTH 40:CLS:COLOR 15,1,1
720 J=0
730 LET P=P+1
740 IF P=13 THEN 1810
750 PRINT:PRINT
760 PRINT"      1. water"
770 PRINT"      2. sugar"
780 PRINT"      3. potassium ions"
790 PRINT"      4. sodium ions"
800 PRINT"      5. chloride ions"
810 PRINT"      6. waste"
820 PRINT"            (primarily ammonia compounds)"
830 PRINT     "Choose the number of the item ";
840 PRINT"which you wish to change."
850 INPUT     X
860 LET H=H+400
870 LET S=S-4
880 LET K=K-2
890 LET N=N+2
900 LET W=W+4
910 IF X=1 THEN 970
920 IF X=2 THEN 1210
930 IF X=3 THEN 1310
940 IF X=4 THEN 1380
950 IF X=5 THEN 1480
960 IF X=6 THEN 1510
970 PRINT     "What is the number of water molecules"
980 PRINT     "that you want removed";
```

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```
990 INPUT     A
1000 IF AH THEN 1030
1010 PRINT     "YOU DONT HAVE THAT MANY!"
1020 GOTO 970
1030 LET H=H-A
1040 PRINT     "Energy is used here to get rid of water"
1050 LET S=S-4
1060 PRINT     " "
1070 PRINT     "PRESENT CONDITIONS ARE AS FOLLOWS:"
1080 PRINT
1090 PRINT    "POTASSIUM IONS--";K,"WASTE MOLECULES--";W
1100 PRINT    "SODIUM INS--";N,"WATER MOLECULES--";H
1110 PRINT    "CHLORIDE IONS--";C,"SUGAR MOLECULES--";S
1120 PRINT     " "
1130 IF Hp THEN 1610
1140 IF H8000 THEN 1610
1150 IF S THEN 1640
1160 IF S35 THEN 1640
1170 IF K( THEN 1690
1180 IF K60 THEN 1690
1190 IF W10 THEN 1730
1200 GOTO 720
1210 PRINT     "How many sugar molecules do you want"
1220 INPUT     B
1230 IF B    THEN 1270
1240 PRINT     "The solution cannot offer you that many"
1250 PRINT     "at once!"
1260 GOTO 1210
1270 LET S=S+B
1280 PRINT     "Energy is needed for pinocytosis!"
1290 LET S=S-3
1300 GOTO 1060
1310 PRINT"Potassium ions are regulated by active"
1320 PRINT"transport. More specifically they are"
1330 PRINT"regulated by the sodium pump, which "
1340 PRINT"is for every sodium ion pumped out, one"
1350 PRINT"potassium ion is gained on the inside"
1360 PRINT"of the membrane."
1370 GOTO 730
1380 PRINT     "How many sodium ions do you want removed"
1390 INPUT     D
1400 IF D 20 THEN 1430
1410 PRINT     "YOU DO NOT HAVE THAT MANY!!!"
1420 GOTO 1380
1430 LET N=N-D
1440 LET K=K+D
```

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```
1450 PRINT     "Energy is needed for active transport!!"
1460 LET S=S-2
1470 GOTO 1060
1480 PRINT     "YOU HAVE LITTLE OR NO CONTROL OR NEED TO"
1490 PRINT     "CHANGE THE CHLORIDE ION CONCENTRATION!!"
1500 GOTO 730
1510 PRINT     "How many waste molecules do you want to"
1520 PRINT     "get rid of"
1530 INPUT     E
1540 IF EW-3 THEN 1570
1550 PRINT     "YOU DO NOT HAVE THAT MANY TO GET RID OF!"
1560 GOTO 1510
1570 LET W=W-E
1580 PRINT     "Energy is needed to get rid of waste!"
1590 LET S=S-4
1600 GOTO 1060
1610 PRINT     "***CRISIS***"
1615 PRINT "ADJUST WATER CONCENTRATION IMMEDIATELY!!"
1620 LET J=J+1
1630 GOTO 1760
1640 PRINT     "***CRISIS***"
1645 PRINT "ADJUST SUGAR CONCENTRATION IMMEDIATELY!!"
1650 IF S0 THEN 1670
1660 PRINT     "YOU ARE BURNING PROTOPLASM!"
1670 LET J=J+1
1680 GOTO 1760
1690 PRINT     "***CRISIS***"
1695 PRINT "ADJUST POTASSIUM ION CONCENTRATION "
1700 PRINT     "                         IMMEDIATELY!!"
1710 LET J=J+1
1720 GOTO 1760
1730 PRINT     "***CRISIS***"
1735 PRINT "ADJUST WASTE CONCENTRATION IMMEDIATELY!!"
1740 LET J=J+1
1750 PRINT
1760 IF J THEN 730
1770 PRINT     "YOU DID NOT FUNCTION WELL AS A"
1780 PRINT"CELL MEMBRANE."
1790 PRINT     "***YOUR CELL IS NOW DEAD********"
1800 GOTO 1890
1810 WIDTH 80:CLS:PRINT      "***VERY GOOD*** "
1815 PRINT "YOU HAVE MAINTAINED THE CELL LONG"
1820 PRINT     "ENOUGH TO REACH MATURITY. THE CELL IS NOW ABOUT TO"
1830 PRINT     "REPRODUCE. DO YOU WISH TO TRY TO KEEP ONE OF THE "
1840 PRINT     "DAUGHTER CELLS ALIVE "
1850 INPUT     A$:A$=LEFT$(A$,1):IF A$="y" OR A$="Y" THEN 1970
```

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```
1860 LET P=0
1870 REM
1880 REM                    SUBROUTINES
1890 END
1900 PRINT     "DO YOU WISH TO SEE AN EXPLANATION OF CONCENTRATIONS"
1910 PRINT     "AND RULES";
1920 INPUT A$:A$=LEFT$(A$,1):IF A$="Y" OR A$="y" THEN 490
1930 IF A$="n" OR A$="N" THEN 1950
1940 END
1950 WIDTH 40:COLOR 15,1,1:CLS
1960 GOTO 720
1970 WIDTH 40:CLS:P=0:GOTO 720
```

BEER (BASIC program for Beer-Lambert Law)

```
10 REM************************************************************
20 REM FILE NAME:                BEER.BAS                    W.Heidcamp
30 REM************************************************************
40 REM
50 REM ** R1$-R4$=RESPONSES TO INPUT PARAMETERS
60 REM ** M=SLOPE OF    REGRESSION = ABSORPTIVITY       B=Y INTERCEPT
70 REM ** N = NUMBER OF DATA POINTS IN STANDARD CURVE
80 REM ** SX,SY,SX2,SXY ARE SUMS OF XY ,X^2,X*Y RESPECTIVELY
90 REM ** DIMENSIONED VARIABLES X = CONCENTRATION, Y = ABSORBANCE
100 REM************************************************************
110 REM
120 DIM X(20), Y(20), YT(20), X2(20), XY(20), Y2(20)
130 REM
140 KEY OFF: SCREEN 0:WIDTH 80:COLOR 14,1,1:CLS:LOCATE 1,1
150 PRINT "THIS PROGRAM WILL COMPUTE THE EXTINCTIOIN COEFFICIENT"
160 PRINT "AND USE THIS # TO COMPUTE THE CONCENTRATIONS OF UNKNOWNS"
170 PRINT:PRINT
180 PRINT "CALCULATIONS ARE BASED ON BEER-LAMBERT LAW A=ecl"
190 PRINT "                    WHERE        A = ABSORBANCE"
200 PRINT "                                 e = ABSORBTIVITY (EXTINCTION COEFFICIENT)
210 PRINT "                                 c = CONCENTRATION
220 PRINT "                                 l = LENGTH OF OPTICAL PATH (1 cm FOR SPEC 20)
230 PRINT "     ":LOCATE 19,1
240 REM    CALCULATE ABSORBTIVITY FOR STANDARD
250 CLS:LOCATE 1,1
260         PRINT "IT WILL FIRST BE NECESSARY FOR YOU TO ENTER YOUR DATA"
270         PRINT "FROM THE STANDARDIZATION."
280         PRINT "HOW MANY DATA POINTS IN YOUR STANDARD ";
290         INPUT N
300         PRINT:PRINT "IS YOUR DATA     TRANSMITTANCE (T) OR ABSORBANCE (A)";
310         INPUT R2$
320         IF R2$ = "A" THEN 430:IF R2$ = "a" THEN 430
```

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```
330       IF R2$    "T"    THEN 300
340       PRINT "PLEASE ENTER THE DATA IN THE FOLLOWING    ORDER:"
350             PRINT "CONCENTRATION, TRANSMITTANCE"
360             PRINT "INCLUDE A COMMA BETWEEN THE TWO VALUES!":PRINT
370                FOR I = 1 TO N
380                PRINT "DATA POINT NUMBER ";I;"      ";
390                INPUT X(I),YT(I)
400                 Y(I) = LOG(100/YT(I))/LOG(10)
410            NEXT I
420       GOTO 490
430          PRINT "CONCENTRATION, ABSORBANCE"
440          PRINT "BE SURE TO SEPARATE THE TWO VALUES BY A COMMA!"
450               FOR I = 1 TO N
460               PRINT "DATA POINT NUMBER ";I;"      ";
470               INPUT X(I),Y(I)
480       NEXT I
490   REM *************************    PRINT DATA AS ABSORBANCE
500   CLS
510    PRINT:PRINT "YOUR DATA (AS ENTERED OR CONVERTED)":PRINT
520    PRINT "CONCENTRATION" TAB(30) "ABSORBANCE"
530    PRINT "--------------" TAB(30) "----------"
540    FOR I = 1 TO N
550    PRINT USING "### ";X(I);:PRINT TAB(34);:PRINT USING "#.###";Y(I)
560    NEXT I
570   PRINT
580   REM ***************    CALCULATE THE LINEAR REGRESSION FOR THE DATA
590 SX=0: SY=0: SX2=0: SXY=0
600     SY2=0
610     FOR I=1 TO N
620        SX=SX+X(I)
630        SY=SY+Y(I)
640        X2(I)=X(I)^2
650        SX2=SX2+X2(I)
660        Y2(I)=Y(I)^2
670        SY2=SY2 + Y2(I)
680        XY(I)=X(I)*Y(I)
690        SXY=SXY+XY(I)
700   NEXT I
710   REM ****************************    CALCULATE THE SLOPE OF THE LINE    M
720    M=(SXY-(SX*SY/N))/(SX2-(SX^2/N))
730   REM ****************************    CALCULATE THE Y INTERCEPT
"B"
740   B=(SY-(M*SX))/N
750 REM ****************************    CALCULATE S.DEVIATIONS FOR X AND Y
760       SDX = SQR((SX2-(SX^2/N))/(N-1))
770       SDY = SQR((SY2-(SY^2/N))/(N-1))
```

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```
780 REM ********************    CALCULATE THE CORRELATION COEFFICIENT    "CC"
790       CC = M*SDX/SDY
800    PRINT "YOUR DATA FITS THE GENERAL EQUATION OF Y=MX + B, WHERE"
810    PRINT "M AND B ARE THE SLOPE AND Y INTERCEPT RESPECTIVELY."
820    PRINT "Y = ";:PRINT USING "#.####";M;
825    PRINT " X ";:PRINT USING "+##.####";B
830    PRINT:PRINT "THE CORRELATION COEFFICIENT, R = ";CC
840    PRINT
843    PRINT "THE SLOPE M (";:COLOR 15,1,1:PRINT USING "#.#####";M;:COLOR 14,1,1
847    PRINT ") IS THE VALUE FOR THE EXTINCTION COEFFICIENT"
850    PRINT
853 PRINT "PRESS SHIFT/PrtSc FOR A PRINTOUT OF THIS INFORMATION":PRINT
860    END
```

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Appendix E: Image Analysis

Computers and video recorders have been combined in a powerful combination for advanced histochemical work. A video camera can be attached to a microscope and coupled to a frame-grabber board within a personal computer. The image can then be captured and displayed on a video monitor in either black & white, color, or pseudocolor. The latter is an enhancement feature of the system which allows assignment of a color to a chosen gray scale of a black & white image. That is, the color is not real, but an assigned value, determined by the operator or the computer system. Since the eye can detect more colors than tones of gray, it increases the ability to visually distinguish subtle tone differences.

A sample system suitable for the undergraduate laboratory is represented by the CUE 2 system manufactured by Olympus. The system consists of:

- CCD video camera and power supply
- Frame grabber board
- Computer equipped with math coprocessor
- Computer mouse or pointing device
- Software
- Cables
- Optional dot matrix printer

Image analysis systems used to be too expensive for routine work, but the price is steadily decreasing. A generic system can actually be pieced together from commercially available components for under $10,000. A fully integrated package will cost slightly more, and a real time system (one which captures and analyzes the image as it is generated) can increase the price by a factor of 10. You get a lot of power for the investment, however.

Image analysis begins with a digital capturing of the image as data. In an intermediate system suitable for cell work, the image is detected with 256 gray levels (the human eye can detect only 9) and displayed with a resolution of either 256 x 256 or 512 x 512 pixels. A pixel is an abbreviation for picture elements, and represents the dots on a screen, or more significantly, the number of information points in the image. A 512 x 512 pixel image thus contains 262,144 pieces of information. Each piece of information will in turn be stored as 8, 16 or 32 bits in computer memory, depending on the computer system attached. An 8 bit machine (IBM PC or equivalent) will satisfy the low end of this scale, a 16 bit (IBM AT or 286 clone) the intermediate, while the upper end will require a significantly faster computer (386 based, or minicomputer). For an undergraduate laboratory (and most research work), the information stored in the low end system (256 x 256, 8 bit) is sufficient, with the high end reserved for analysis of true color and/or real time images. A display resolution of 512 x 512 pixels makes the image sharper appearing and easier to work with.

Once the image is acquired in digital form, it becomes data which can be manipulated for changes in the display image, or for statistical purposes. The operational capabilities of the CUE 2 system, as listed in Figure E-2, demonstrate many of these features.

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Software options for image analysis include packages for densitometry (replacing microspectrophotometers), 3-D construction of serial sections, production of stereo images, autoradiographic analysis, planar morphometry including linear analysis (line length, width and angles) in addition to the features listed for the basic system.

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Appendix F: Centrifugation

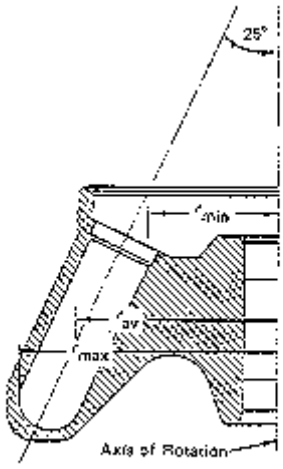
The single most important advance in the use of centrifugal force to separate biologically important substances. was the coupling of mechanics, optics and mathematics by T. Svedberg and J.W. Williams in the 1920's. They initiated the mathematics and advanced the instrumentation. 1 to a point where it was possible to prove that proteins were large molecules that could be weighed in a centrifuge. 2. In honor of that work, the value for a molecule's (or organelle's) sedimentation velocity in a centrifugal field is known as its Svedberg constant or S value for short.

The instrumentation has progressed quite far since the early work of Svedberg and Williams. Today, any technique employing the quantitative application of centrifugal force is known as ultracentrifugation. The design of the basic instruments, the rotors and the optical systems for measurement are too extensive to cover in this volume. For our purposes, we will concentrate on two types of rotor, and a few selected parameters to be measured.

ROTORS

Table F.1. Rotor Characteristics

Figure F.1. Cross section of Sorvall SS-34 rotor



Rotors for a centrifuge are either fixed angles , swinging buckets , continuous flow, or zonal, depending upon whether the sample is held at a given angle to the rotation plane, is allowed to swing out on a pivot and into the plane of rotation, designed with inlet and outlet ports for separation of large volumes, or a combination of these. Figure F.1 demonstrates the characteristics of each of these.

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Fixed angles generally work faster; substances precipitate faster in a given rotational environment, or they have an increased relative centrifugal force for a given rotor speed and radius. They also have few (or no) moving parts on the rotor itself and thus have virtually no major mechanical failures, other than potential metal stress, which all rotors undergo. These rotors are the work-horse elements of a cell laboratory, and the most common is a rotor holding 8 centrifuge tubes at an angle of 34 ° C from the vertical (such as the Sorvall SS-34 rotor or the Beckman JA-20). Figure F.1 presents a cross- sectional diagram of the Sorvall SS-34.

Swinging bucket rotors (also known as horizontal rotors) have the advantage that there is usually a clean meniscus of minimum area. In a fixed angle rotor, the materials are forced against the side of the centrifuge tube, and then slide down the wall of the tube. This action is the primary reason for their apparent faster separation, but also leads to abrasion of the particles along the wall of the centrifuge tube. For a swinging bucket, the materials must travel down the entire length of the centrifuge tube and always through the media within the tube. Since the media is usually a viscous substance, the swinging bucket appears to have a lower relative centrifugal force, that is it takes longer to precipitate anything contained within. If, however, the point of centrifugation is to separate molecules or organelles on the basis of their movements through a viscous field, then the swinging bucket is the rotor of choice. Moreover, if there is a danger or scraping off an outer shell of a particle (such as the outer membrane of a chloroplast), then the swinging bucket is the rotor of choice. Most common clinical centrifuges 1 have swinging buckets. Since the buckets are easy to interchange, this type of rotor is extremely versatile. Its major drawback is the number of moving parts which are prone to failure with extended use.

Nearly all cell biology laboratories will have several examples of fixed angle and horizontal rotors. While the sample volumes of these rotors can be significant, they are limiting. To overcome this limitation, a continuous flow centrifuge can be used. Limnologists often employ such a device to separate plankton from gallons of lake water. Cell biologists employ zonal rotors for the large scale separation of particles on density gradients. Zonal rotors can contain up to 2 liters of solution and can work with tissue samples measured in ounces (or even pounds). The rotors are brought up to about 3000 RPM while empty, and the density media and tissues are added through specialized ports. This type of rotor has a distinct preparative advantage over the gradient capacity of more typical rotors.

ROTOR TUBES

In using either a fixed angle or swinging bucket rotor, it is necessary to contain the sample in some type of holder. Continuous and zonal rotors are designed to be used without external tubes.

For biological work, the tubes are divided into functional groups, made of regular glass, Corex glass, nitrocellulose, or polyallomer. Regular glass centrifuge tubes can be used at speeds below 3,000 RPM, that is in a standard clinical centrifuge. Above this speed, the xg forces will shatter the glass.

A special high speed glass with the tradename of Corex (Corning Glass Works) has been developed to handle speeds up to 15-18,000 RPM. These tubes can be used in most routine cell organelle preparations, if, and only if, the proper adapters are also used within the centrifuge rotors.

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These tubes are relatively expensive (about $3.50 each) and should never be used for any purpose other than the centrifuge. Any tubes with scratches or chips should be disposed of immediately. These high- speed glass tubes will shatter above 18,000 RPM.

For work in the higher speed ranges, centrifuge tubes are made of plastic or nitrocellulose. Preparative centrifuge tubes are made of polypropylene (sometimes polyethylene) and can withstand speeds up to 20,000 RPM. These tubes should be carefully examined for stress fractures before use. A tube with a fracture will hold fluids before centrifugation, but the cracks will open under centrifugal force.

Nitrocellulose are inexpensive and used for most ultracentrifugation. They are meant to be used only once and then discarded. Repeated use increases the chance of tube collapse due to internal molecular stress within the tube walls. There is no way to pre-determine this, so it is best to always use a new tube for ultracentrifugation. Nitrocellulose also becomes less flexible with age, and the purchase date for all tubes should be noted. Tubes older than 1 year should be discarded. A centrifuge tube is inexpensive when compared to the loss of time and materials for a typical ultracentrifuge run.

Polyallomer tubes are re-usable, more expensive, and slippery. Molecules will slide down the walls of these tubes more easily, and thus are the tubes of choice for precipitation centrifugations. They are also more chemically inert.

Modern day ultracentrifuges can generate forces in excess of 300,000 times that of gravity, forces sufficient to overcome the very cohesion of most molecules (including the metal of the rotor). The force is usually given as some value times that of gravity.

The centrifugal force is dependent upon the radius of the rotation of the rotor, the speed at which it rotates, and the design of the rotor itself (fixed angle, vs swinging bucket). Rotor speed and design can be held constant, but the radius will vary from the top of a centrifuge tube to the bottom. If a measurement for the radius is taken as the mid-point, or as an average radius, and all forces are mathematically related to gravity, then one obtains a relative centrifugal force, labeled as xg. Centrifugation procedures are given as xg measures, since RPM and other parameters will vary with the particular instrument and rotor used. Relative Centrifugal Force is a constant that is independent of the apparatus used.

Figure F.2 presents a Nomogram for calculation of R.C.F. for a given radius and RPM. A simple formula for calculating this value is:

$$RCF = 1.12r\,(RPM/1000)^2$$

where r = radius in millimeters
RPM = revolutions per minute

The difficulty with using the formula is establishing the value for r. Typically, there are three r values given (by the manufacturer) for a rotor: the maximum, minimum and average r. These correspond to the distances from the center of rotation to the bottom, top and middle of the sample tube.

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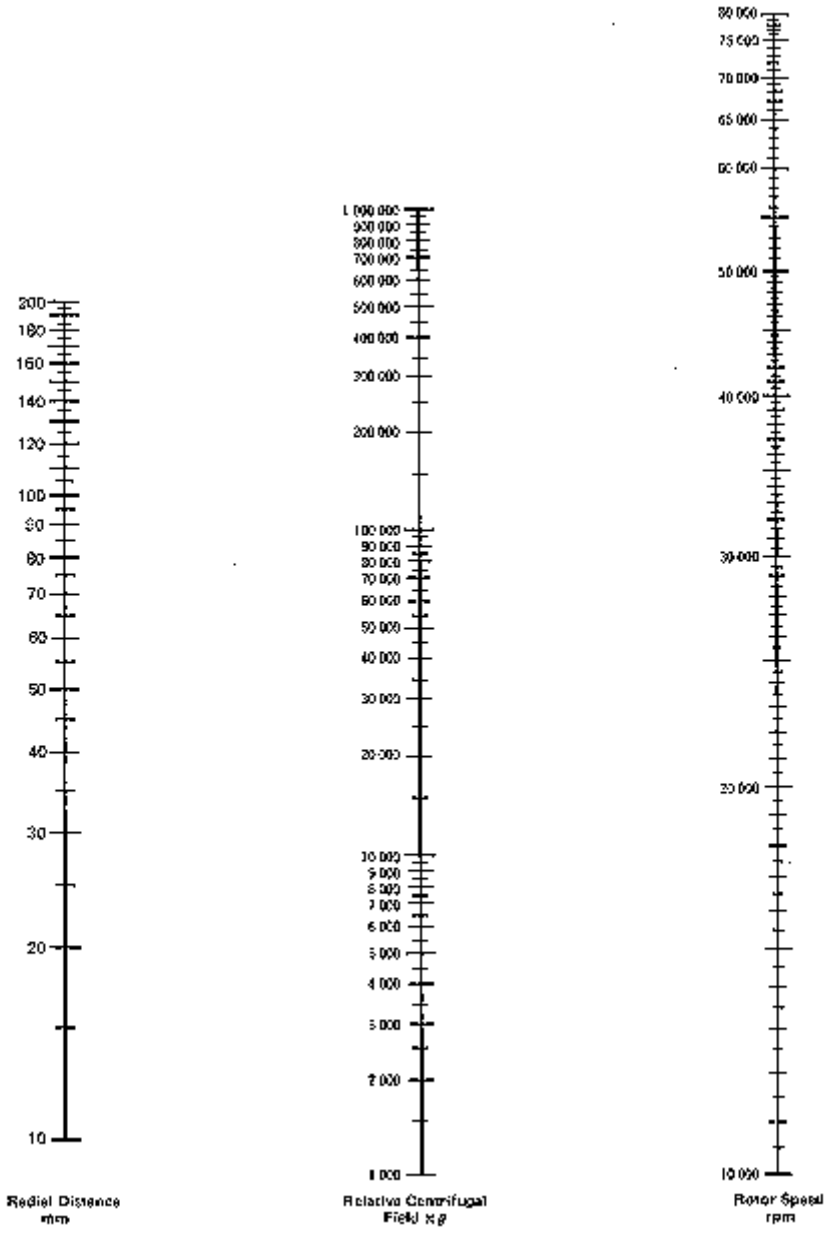
RELATIVE CENTRIFUGAL FORCE

Figure F.2. Nomogram for R.C.F.



If the density and viscosity of the medium are known, as well as the density of a given particle, then the time needed to completely sediement a particle can be determined by the formula:

$T = ((D-L)/(D+L))*(N/(d^2(g-p)S^2))$

where T = time in minutes

D = radial distance in cm for rmax

L = radial distance to meniscus

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N = viscosity of the fluid medium
g = density of the fluid medium
p = density of the particle to sediment
d = diameter of the particle in cms.
S = rotational velocity in RPM

ROTOR COMPARISONS

When separating a particle, it is convenient to be able to compare one rotor's design characteristics with those of a differing rotor. A procedure may be given as centrifuging for 20 minutes at 15,000 RPM in a Sorvall SS34 rotor. The problem then becomes one of how do you equate that to a Beckman JA-20 rotor?

Working with the maximum RCF for each rotor, the conversion can be made with the following equation:

$t_1 = (t_2 \times RCF_2) / RCF_1$
where t_1 = run time needed for the Beckman rotor t_2 = run time specified in the procedure
RCF_1 = RCF of JA-20 rotor at maximum speed
RCF_2 = RCF specified in the procedure
(calculated if procedure in RPM)

This equation can be altered somewhat to be even more useful. The efficiency of rotors can be compared for any given task. Each rotor is given a value for k (the clearing constant, or clearing factor), which is an estimate of the time (in hours) required to pellet a particle of known sedimentation coefficient at the maximum speed of the rotor. [4] The lower the value of k, the shorter the time required to sediment a given particle. The relationship is given by

$t = k / s_{20,w}$
k = clearing factor expressed in hour-Svedbergs
$s_{20,w}$ = Sedimentation Coefficient in water at 20 ° C
expressed in Svedbergs

It is possible to compute k, but it is easiest to use the manufacturers values, which can be looked up in tables such as those in Table F.1.

$k = (253{,}300)[\ln(r_{max}/r_{min})]/(\text{Rotor angular velocity}/1000)^2$

Using tabulated k values, however, the comparison of rotors is even easier, as the equation above becomes

$t_1 = k_1 t_2 / k_2$
where the t and k values are the time and k factor for each rotor.

By substituting this equation, you can also determine the time of centrifugation for any change in rotor speed. [5]

For a Sorvall SS-34, for example, at 20,000 RPM (k=402), the time required to sediment a particle of100 S, in water at 20 ° C is calculated by:

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t = 402/100or 4.02 hours (4 hrs, 1 min)

Table F.2 presents the k values for the Sorvall SS-34 rotor and for the Sorvall GSA rotor. Since these two rotors will be used throughout the remainder of the course, reference should be made to this table in any future centrifugation work, when another rotor is substituted.

ROTOR SAFETY

There are a number of safety precautions that must be adhered to when using any centrifuge and rotor.

Rotor Failure:

All rotors are subject to stress and with time will undergo metal fatigue. This is a given, and consequently, a detailed history of the rotor use should be kept. This is usually not done with clinical centrifuges, but is an absolute for an ultracentrifuge rotor.

EVERY USE OF AN ULTRACENTRIFUGE ROTOR MUST BE RECORDED IN THE CENTRIFUGE LOG. ABSOSLUTELY NO EXCEPTIONS!

After a period of use, each rotor will in turn be derated , that is its maximum RPM will be lowered. The Beckman rotors may contain optical speed control rings at their base - be sure they are present, and clean before use. These devices will strobiscopically monitor the maximum speed that a rotor can be used at. They are replaced as the rotor is derated.

By far the most common cause of rotor failure is corrosion stress. Salts, highly alkaline detergents and of course corrosive acids and alkali's will cause decomposition of the coatings on aluminum rotors, which in turn will concentrate stress and eventually result in cracks and total rotor failure. Titanium rotors are more corrosion resistant, but more expensive. Ultracentrifuge rotors are expensive (in excess of $6,000 each on average) and can be potentially hazardous. At the forces generated in an ultracentrifuge, a rotor failure is the equivalent of a small bomb.

THEREFORE THE FOLLOWING RULES MUST BE OBSERVED.

1. Before running a centrifuge, check the classification decal on the centrifuge to ensure that the rotor is safe to use in the centrifuge at hand.
2. Never use an alkali detergent on a rotor (most are highly alkaline - be sure to check before use).
3. Always clean and completely dry the rotor after every use. Any spilled materials, especially salts and corrosive solvents must be removed immediately with running water. Fixed angle rotors are stored upside down, to drain after thorough cleaning and rinsing. Swinging buckets have only the buckets cleaned and dried, and stored inverted and with the caps removed. NEVER immerse the rotor portion of a swinging bucket rotor. Inevitably the linkage pins will rust, as it is virtually impossible to remove all fluids from them.
4. Be especially careful not to scratch the surface of a rotor or bucket. Use plastic brushes only. Normal wire brushes will scratch the anodized surface of aluminum rotors which will increase the likelihood of corrosion. The anodized layer is extremely thin and is the main defense against corrosion of an aluminum rotor.
5. Always use the proper centrifuge tube. Glass tubes are used in clinical centrifuges only. High Speed

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Corex tubes can be used up to 15,000 RPM (in SS34 rotor) IF there are no scratches or imperfections in the glass, and if the proper rubber or plastic adapter is employed. All ultracentrifugation use employs nitrocellulose or polyallomer tubes. Nitrocellulose ages and will collapse in a strong centrifugal field if old.

6. Always fill the centrifuge tubes to the proper level. (usually full to within 1/2 inch of the top). The tubes are thin walled and will collapse if improperly filled.
7. Always balance the rotor properly. Use a precision scale for most work. Always balance the tube with a medium that is identical to that being centrifuged, i.e. do not balance an alcohol solution with water, or a dense sucrose solution with water only -- the distribution of the densities will be incorrect. For swinging buckets, be sure the buckets are weighed with their caps in place, that the seals are intact and that the caps are secure. Be careful in the placement of tubes within a rotor to ensure proper balance - check the manufacturers guides for complex rotors that hold multiple tubes.
8. Ensure that the rotor is properly seated within the centrifuge. For swinging buckets, ensure that they are hanging properly - Double or Triple check! For preparative rotors, be sure the rotor cover is in place and properly screwed down, where appropriate. NEVER use a rotor without its lid, when one is supplied - the screw actually holds the rotor to the motor shaft.
9. Check that the centrifuge chamber is clean, defrosted and that all membranes or measuring devices are intact and functional (Beckman speed and temperature controls) and that the lid is securely closed.
10. Adjust acceleration rates, deceleration rates, temperature and RPM controls as appropriate. Set brake on or off as appropriate and check vacuum level where appropriate.
11. Start the centrifuge and set the timer. Do not attempt to open the centrifuge until the rotor has come to a complete stop.
12. Before opening the centrifuge, record the appropriate information in the centrifuge log.

Note: If properly balanced and used, the rotor should accelerate smoothly and with a constant change in the pitch of the motor sound. Any vibrations, or unusual sounds should cause the cessation of operation IMMEDIATELY by the operator. **NEVER** leave the centrifuge until you are certain that it has reached its operating speed and is functioning properly. All rotors go through a minor vibration phase when they first start. There will be a minor flutter when the rotor reaches this vibration point - do not confuse this with a serious vibration caused by imbalance. If in doubt, halt the centrifuge and get assistance.

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Appendix G: Spectrophotometry

Figure G.1 Electromagnetic Radiation Spectrum

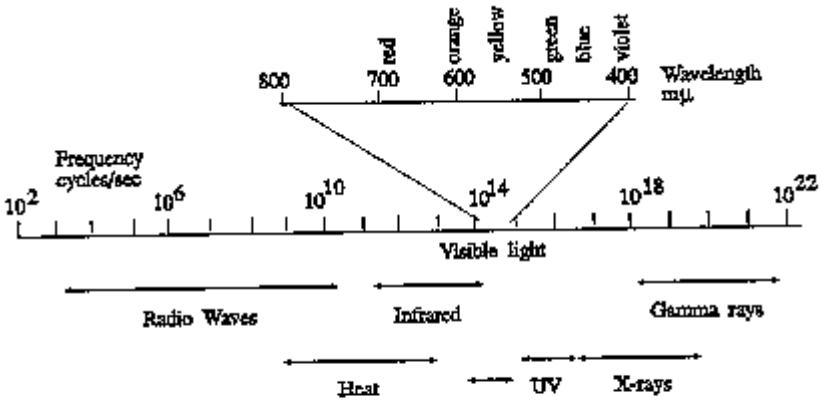

Figure G.2. Schematic light transmission

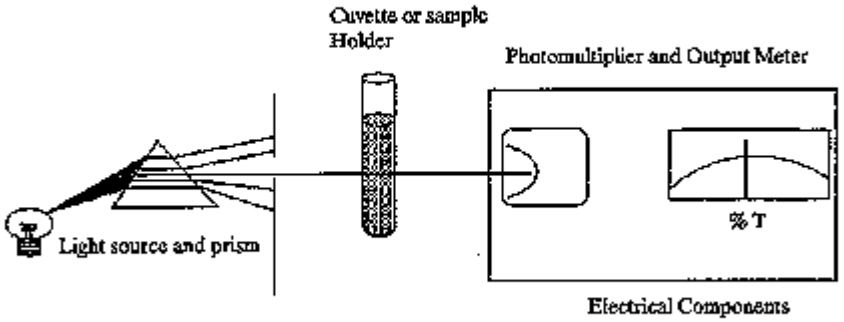

Figure G.2. Schematic light transmission.

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Figure G.3.Use of the Spec 20

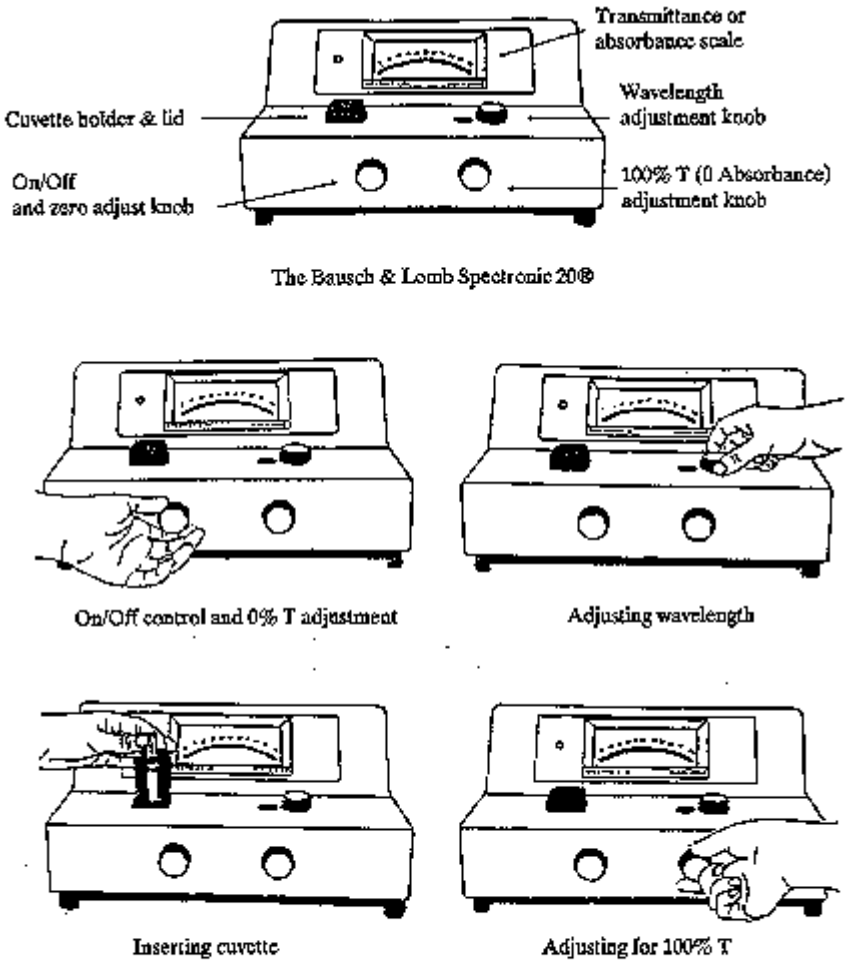

Figure G.3. Use of the Spec 20.

A spectrophotometer or colorimeter makes use of the transmission of light through a solution to determine the concentration of a solute within the solution. A spectrophtometer differs from a colorimeter in the manner in which light is separated into its component wavelengths. A spectrophotometer uses a prism to separate light and a colorimeter uses filters.

Both are based on a simple design of passing light of a known wavelength through a sample and measuring the amount of light energy that is transmitted. This is accomplished by placing a photocell on the other side of the sample. All molecules absorb radiant energy at one wavelength of another. Those that absorb energy from within the visible spectrum are known as pigments. Proteins and nucleic acids absorb light in the ultraviolet range. The following figure demonstrates the radiant energy spectrum with an indication of molecules which absorb in various regions of that spectrum.

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The design of the single beam spectrophotometer involves a light source, a prism, a sample holder and a photocell. Connected to each are the appropriate electrical or mechanical systems to control the illuminating intensity, the wavelength, and for conversion of energy received at the photocell into a voltage fluctuation. The voltage fluctuation is then displayed on a meter scale, is displayed digitally, or is recorded via connection to a computer for later investigation.

Spectrophotometers are useful because of the relation of intensity of color in a sample and its relation to the amount of solute within the sample. For example, if you use a solution of red food coloring in water, and measure the amount of blue light absorbed when it passes through the solution, a measureable voltage fluctuation can be induced in a photocell on the opposite side. If now the solution of red dye is diluted in half by the addition of water, the color will be approximately 1/2 as intense and the voltage generated on the photocell will be approximately half as great. Thus, there is a relationship between the voltage and the amount of dye in the sample.

Given the geometry of a spectrophotometer, what is actually measured at the photocell is the amount of light energy which arrives at the cell. The voltage meter is reading the amount of light TRANSMITTED to the photocell. Light transmission is not a linear function, but is rather an exponential function. That is why the solution was APPROXIMATELY half as intense when viewed in its diluted form.

We can however monitor the transmission level and convert it to a percentage of the amount transmitted when no dye is present. Thus, if 1/2 the light is transmitted, we can say that the solution has a 50% Transmittance. Note that it is always relative to a solution containing no dye.

Transmittance is the relative percent of light passed through the sample.

What makes all of this easy to use, however, is the conversion of that information from a percent transmittance to an inverse log function known as the Absorbance (or Optical Density).

The Beer-Lambert Law

Definiton

Absorbance: The negative $\log_{10}$ of the transmittance.

$A = - \log_{10} T$ EQUATION G.1

This value is more useful in spectrophotometry than transmittance, because of plot of absorbance vs concentration yields a straight line. A plot of transmittance vs concentration is an exponential. The - log calculates the inverse of transmittance, so that absorbance increases with increasing concentration. Transmittance would decrease as we increased the amount of red dye in our example. The relationship of Absorbance to concentration was shown by two biochemists to follow the equation for a straight line, y = mx +b, where m is the slope of the line and b is the y intercept. If the measurement is made in such a way that b = 0 (that is, a solution containing no dye has no absorbance), and if we substitute Absorbance for y, concentration for x, and variant for m, we arrive at the formulation of the Beer-Lambert Law:

$A = \varepsilon C$

where A = absorbance

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C = concentration

ε = the extinction coefficient

Note that variant is equal to the slope of the straight line which will result from a plot of absorbance (y axis) vs concentration (x axis).

To use a spectrophotometer it is necessary to establish a known series of dilutions containing known quantities of a solute. One of these will contain no solute and is known as the blank . It is used to adjust the instrument to read 100% transmittance or 0 absorbance. In use, a 0% transmittance value (infinite absorbance) is established by placing a curtain between the light source and the photocell. Electronic control is then exerted so that the meter will read 0% Transmittance on its scale. The blank sample (containing no solute or dye) is inserted, the curtain opened and the meter readjusted to read 100% transmittance. All other measures are then made by merely inserting the samples into the light path and measuring the % transmittance. Most spectrophotometers have a built in means of direct conversion of this reading to absorbance.

After recording the absorbance for a series of standards, a plot is made of the absorbance value (y axis) vs the concentration (x axis). The slope of the line is the extinction coefficient.

Note that this may be computed directly by rearrangement of the Beer-Lambert law to

$\varepsilon = A/C$ EQUATION G.2

This value can be calculated for each reading and the average taken as the value of variant. Remember that this value is a constant. Thus, once calculated, it can subsequently be used to determine an unknown concentration by one more rearrangement of the Beer-Lambert law

$C = A/\varepsilon$ EQUATION G.3

Any measured value of A can be readily converted to a corresponding concentration merely by dividing the absorbance by ε.

The use of the Beer-Lambert law is easy to visualize with red food coloring. It is not as easy to visualize, but none the less, just as accurate to measure wavelengths of light which are not visible. Either infra red or ultraviolet can be used. UV is more useful to biologists since many molecules (all proteins and nucleic acids) absorb ultraviolet light. The only changes that need to be made is the use of quartz cuvettes instead of glass tubes. Glass absorbs UV light and thus is inappropriate for use in a UV spectrophotometer. An instrument capable of using visible light (usually with a tungsten or halogen lamp source) and UV light is known as a UV/Vis Spectrophotometer.

OPERATION OF B&L SPEC. 20

The most commonly encountered spectrophotometer is one manufactured by Bausch and Lomb and known as the Spec 20. The 20 refers to the band size of light that it is capable of producing. If the instrument is adjust to a wavelength of 730, for example, it actually transmits light from 720 nm to 740 nm. Thus, it is not as precise or refined as instruments designed for research purposes where the wavelength may be controlled to a fraction of a nanometer. It is, however, the standard workhorse instrument found in nearly every lab.

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1. Turn on the spectrophotometer and allow 10 minutes for warm up of the instrument before use.
2. Adjust the wavelength to that specificied for the procedure you are using.
3. Be sure the cover is closed on the cuvette holder and use the left knob on the front panel to adjust the dark current such that the meter is reading 0 transmittance. At this point, you are simply adjusting the internal electronics of the instrument to blank out any residual currents. This adjusts the lower limit of measurements. It establishes that no light is equivalent to 0 transmittance or infinite absorbance.
4. Insert a clean cuvette containing the blank into the holder. Be sure that the tube is clean, free of finger prints and that the painted line marker on the tube is aligned with the mark on the tube holder. Close the top of the tube holder. The blank for this exercise is the solution containing no dopachrome, but all other chemicals. The amount of solution placed in the cuvette is not important, but is usually about 5 ml. It should approximately reach the bottom of the logo printed on the side of the cuvette.
5. Adjust the meter to read 100% transmittance, using the right knob on the front of the instrument. This adjusts the instrument to read the upper limit of the measurements and establishes that your blank will give a reading of 100% transmittance (0 absorbance).
6. Remove the blank from the instrument and recheck that your 0 transmittance value has not changed. If it does, wait a few minutes for the instrument to stabilize and redo steps 1-5. Periodically throughout the exercise, check that calibration of the instrument is stable by re-inserting the blank and checking that the 0 and 100% T values are maintained.
7. To read a sample, simply insert a cuvette holding your test solution and close the cover. Read the transmittance value directly on the scale.
8. Record the % transmittance of your solution, remove the test tube cuvette and continue to read and record any other solutions you may have.

It is possible to read the absorbance directly, but with an analog meter (as opposed to a digital read out), absorbance estimations are less accurate and more difficult than reading transmittance. Absorbance can be easily calculated from the transmittance value. Be sure that you note which value you measure!

ABSORPTION SPECTRUM:

Analysis of pigments often requires a slightly different use of a spectrophotometer. In the use of the instrument for determination of concentration (Beer-Lambert Law), the wavelength was pre-set and left at a single value throughout the use of the instrument. This value is often given by the procedure being employed, but can be determined by an analysis of the absorption of a solution as the wavelength is varied.

The easiest means of accomplishing this is to use either a dual beam spectrophotometer or a computer controlled instrument. In either event, the baseline must be continously re-read as the wavelength is altered.

To use a single beam spectrophotometer (such as the Spec 20), the machine is zeroed first, the wavelength is set, the blank is adjusted and then the sample is inserted and read. The wavelength is then adjusted up or down by some determined interval, the zero is checked, the blank re-inserted and adjusted, and the sample re-inserted and read. This procedure continues until all wavelengths to be scanned have been read.

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In this procedure, the sample remains the same, but the wavelength is adjusted. Compounds have differing absorbtion coefficients for each wavelength. Thus, each time the wavelength is altered, the instrument must be recalibrated.

A dual beam spectrophotometer divides the light into two paths. One beam is used to pass through a blank, while the remaining beam passes through the sample. Thus, the machine can monitor the difference between the two as the wavelength is altered. These instruments usually come with a motor driven mechanism for altering the wavelength, or scanning the sample.

The newer version of this procedure is the use of an instrument which scans a blank, and places the digitalized information in its computer memory. It then rescans a sample and compares the information from the sample scan to the information obtained from the blank scan. Since the information is digitalized (as opposed to an analog meter reading), manipulation of the data is possible. These instruments usually have direct ports for connection to personal computers, and often have built in temperature controls as well. This latter option would allow measurement of hanges in absorbtion due to temperature changes (known as hyperchromicity). These in turn can be used to monitor viscosity changes, which is related to the degree of molecular polymerization with the sample. For instruments with this capability, the voltage meter scale has given way to a CRT display, complete with graphics and built in functions for statistical analysis.

A temperature controlled UV spectrophotometer capable of reading several samples at pre-programmed time intervals is invaluable for enzyme kinetic analysis. An example of this type of instrument is the Beckman DU-70.

SPECIFIC PROCEDURES:

For routine use, substances to be monitored by spectrophotometry are often reacted with dyes to form a complex that is of another color, usually one easily read within the visible light range, and with precision by an instrument such as the Spec 20.

EXERCISE G.1 BRADFORD PROTEIN ASSAY

MATERIALS

- Lyophilized bovine plasma gamma globulin or bovine serum albumin (BSA)
- Coomasie Brilliant Blue 1
- 0.15 M NaCl
- Spectrophotometer and tubes
- Micropipettes

PROCEDURE (STANDARD ASSAY, 20-150 µ g protein; 200-1500 µ g/ml)

1. Prepare a series of protein standards using BSA diluted with 0.15 M NaCl to final concentrations of 0 (blank = NaCl only), 250, 500, 750 and 1500 µ g BSA/ml. Also prepare serial dilutions of the unknown sample to me measured.
2. Add 100 µ l of each of the above to a separate test tube (or spectrophotometer tube if using a Spec 20).

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3. Add 5.0 ml of Coomasie Blue to each tube and mix by vortex, or inversion.
4. Adjust the spectrophotometer to a wavelength of 595 nm, and blank using the tube from step 3 which contains 0 BSA.
5. Wait 5 minutes and read each of the standards and each of the samples at 595 nm wavelength.
6. Plot the absorbance of the standards vs their concentration. Compute the extinction coefficient and calculate the concentrations of the unknown samples.

PROCEDURE (MICRO ASSAY, 1-10 µ g protein;

1. Prepare standard concentrations of BSA of 1, 5, 7.5 and 10 µ g/ml. Prepare a blank of NaCl only. Prepare a series of sample dilutions.
2. Add 100 µ l of each of the above to separate tubes (use microcentrifuge tubes) and add 1.0 ml of Coomasie Blue to each tube.
3. Turn on and adjust a spectrophotometer to a wavelength of 595 nm, and blank the spectrophotometer using 1.5 ml cuvettes.
4. Wait 2 minutes and read the absorbance of each standard and sample at 595 nm.
5. Plot the absorbance of the standards vs their concentration. Compute the extinction coefficient and calculate the concentrations of the unknown samples.

EXERCISE G.2 LOWRY PROTEIN ASSAY

MATERIALS

- 0.15% (w/v) sodium deoxycholate
- 72% (w/v) trichloroacetic acid (TCA)
- Copper tartrate/carbonate (CTC)
- 20% (v/v) Folin-Ciocalteu reagent
- Bovine Serum Albumin (BSA)
- Spectrophotometer and tubes
- Micropipettes

PROCEDURE

1. Prepare standard dilutions of BSA of 25, 50, 75 and 100 µ g/ml. Prepare appropriate serial dilutions of the sample to be measured.
2. Place 1.0 ml of each of the above into separate tubes. Add 100 µ l of sodium deoxycholate to each tube.
3. Wait 10 minutes and add 100 µ l of TCA to each tube.
4. Centrifuge each tube for 15 minutes at 3,000 xg and discard the supernatant.
5. Add 1.0 ml of water to each tube to dissolve the pellet. Add 1.0 ml of water to a new tube to be used as a blank.
6. Add 1.0 ml of CTC to each tube (including the blank), vortex and allow to set for 10 minutes.
7. Add 500 µ l Folin-Ciocalteu to each tube, vortex and allow to set for 30 minutes.
8. Turn on and zero a spectrophotometer to a wavelength of 750 nm. Use the blank from Step 7 to adjust for 100% T.
9. Read each of the standards and samples at 750 nm.
10. Plot the absorbance of the standards vs their concentration. Compute the extinction coefficient and

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calculate the concentrations of the unknown samples.

NOTES

The Lowry method depends on the presence of tyrosine within the protein to be measured. The standard protein must contain *approximately* the same number of tyrosine residues as the sample, or the procedure will be inaccurate. If there are no tyrosine residues in the sample to be measured, the Lowry method of protein determination is useless, and use should be made of the Bradford assay. In general, the Bradford assay is the method of choice for protein determinations.

EXERCISE G.3 BIURET PROTEIN ASSAY

MATERIALS

- Biuret Reagent
- Bovine serum albumin (BSA)
- Spectrophotometer and tubes

PROCEDURE

1. Prepare standard dilutions of BSA containing 1, 2.5, 5.0, 7.5 and 10 mg/ml protein. Prepare serial dilutions of the unknown samples.
2. Add 1.0 ml of each of the standards, each sample, and 1.0 ml of distilled water to separate tubes. Add 4.0 ml of Biuret reagent to each tube. Mix by vortex.
3. Incubate all of the tubes at 37 ° C for 20 minutes.
4. Turn on and adjust a spectrophotometer to read at a wavelength of 540 nm.
5. Cool the tubes from Step 3, blank the spectrophotometer and read all of the standards and samples at 540 nm.
6. Plot the absorbance of the standards vs their concentration. Compute the extinction coefficient and calculate the concentrations of the unknown samples.

NOTES

The Biuret reaction was one of the first for the determination of protein concentration. It remains as a rapid determination, but is not very accurate. It is useful during protein separation procedures since there are fewer salt interference reactions than with the Bradford or Lowry techniques. The color formed is stable for about 1-2 hours and consequently all spectrophotometer readings must be made as soon as possible after the incubation step.

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Appendix H: Radioactive tracers

The use of radioactive tracers in cell research is an effective and safe means of monitoring molecular interactions. There is simply no other technique which allows the precision and specificity of radioactive tracers.

Radiation is to be taken seriously. At a minimum, its misuse can lead to increased environmental pollution, and at worst can lead to serious long term injury. It can be handled safely, however.

Radioactivity is caused by the spontaneous release of either particulate and/or electromagnetic energy from the nucleus of an atom. Atoms are composed of a positively charge nucleus, surrounded by the negatively charged electrons. In an uncharged atom, the number of orbital electrons equals the number of positively charged protons in the nucleus. In addition, the nucleus contains uncharged neutrons. A proton has a mass of 1.0076 amu (Atomic Mass Units), while a neutron has a mass of 1.0089 amu.

If the mass of a helium nucleus is examined, there is a difference between the expected mass based on its proton and neutron composition, and the actual measured mass. Helium contains two protons and two neutrons in its nucleus, and should have a corresponding mass of 4.0330 amu. It has an actual mass, however, of 5.0028 amu. The difference (0.0302 amu) is the equivalent energy of 28.2 Mev and is known as the binding energy . It would require 28.2 Mev to fuse two protons and two neutrons into a helium nucleus, and the fission of the helium nucleus would yield the same energy.

In addition, the electrons orbit the nucleus with precise energy levels. When the electrons are in their stable orbits, they are said to be in their ground state. If the electrons absorb energy (e.g. from photons), they jump to different, yet characteristic orbits and enter the excited state. The energy difference between a ground state and an excited state can take the form of an electromagnetic radiation.

The number of protons in the nucleus of an atom is called the atomic number, while the number of protons plus neutrons is the mass number. The mass number is approximately equal to the atomic weight. In the representation of an atom used in the periodic table of elements, the atomic number is a subscript written to the left of the letter(s) designating the element, while the mass number is written as a superscript to the left.

The chemical identity of an element is determined by the number of protons in the nucleus of the atom. The number of neutrons may vary, however. Elements sharing the same number of protons, but having different numbers of neutrons are known as isotopes. Hydrogen, for example, has one proton. All nuclei containing one proton are hydrogen nuclei. It may have one, two, or three neutrons. The isotopes of hydrogen would be written as $_1H_1$ $_2H_1$ $_3H_1$ (in all further references, the atomic number subscript 1 is left off for clarity). $_1$H is the most stable form of hydrogen and is therefore the most abundant (99.985% of all forms). $_2$H is also a stable form of hydrogen, but less

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stable than $_{1}H$, and constitutes about 0.015% of the total hydrogen found. It is known as deuterium.

$_{3}H$ is unstable and constitues a very small fraction of the amount of hydrogen available. Termed tritium , this element readily reorganizes its nucleus, and is said to decay. The emission of its sub-atomic particles and energy is therefore known as radioactive decay, or simply radioactivity. Deuterium is a stable, but heavy isotope of hydrogen, tritium is a radioactive isotope of hydrogen.

Note that each of the three will chemically react as hydrogen. This is important for tracer work in cell biology. The substitution of either deuterium or tritium for hydrogen in a molecule will not effect any chemical or physiological changes in the activity of the molecule. Tritium will, however, tag the molecule by making it radioactive.

Radiation emissions have several forms. When an atom reorganizes its sub-atomic structure to a more stable form, it may emit neutrons, protons, electrons, and/or electromagnetic waves (energy). An alpha particle is 2 protons plus 2 neutrons (essentially a helium nucleus). A beta particle is an electron. Gamma rays are electromagnetic energy waves similar to x-rays. The release of sub-atomic particles and energy, resulting in the change of one element to another is known as radioactivity.

Radioactive elements thus, by their very nature, self destruct. The loss of their sub-atomic particles is a spontaneous process, and once it has occurred, the element is no longer radioactive. With time a percentage of all radioactive elements in a solution will decay. Statistically, it is nearly impossible to predict which individual element will radioactively decay, but we can make a prediction about large numbers of the elements. That is, we can say that if we wait 14,000 years, half of the radioactivity in a sample of $_{14}C$ (a radioactive isotope of $_{12}C$) will be lost (1/2 remains). We then say that $_{14}C$ has a half- life of 14,000 years. After a second 14,000 years, half of the remaining half would have been lost, or 3/4 of the original amount. Based on this information, could you predict how long it would take for all radioactivity to have disappeared from the sample?

With a half-life of 14,000 years, radioactive carbon will be around for a very long time. This is why it is used for dating rocks and fossils. If one makes some assumptions about the activity of the carbon when the fossil was formed, and measures the current level, the age of the fossil may be determined.

The amount of radioactive material is measured by how many nuclei decay each second, and this value is known as the activity. It is measured in curies Each radioisotope has three important properties; the type of particles emitted, the particle energy, and the half-life. The energy and kind of decay particle will determine the penetration of the radiation, and therefore determine the degree of shielding necessary to protect the user. The half-life determines both the remaining activity after storage or use, and the time that the isotope must be stored before disposal.

In cell biology, only a few of the many radioactive elements are used routinely. The primary elements used are $_{3}H$ (Tritium), $_{15}C$ (Carbon-14), $_{32}P$ 20(Phosphorus-32), $_{125}I$ 20(Iodine-125) and $_{131}I$ 20(Iodine-137). The characteristics of each of these are given within the following table:

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TABLE H.1 Radioactive Sources and Emission Types

MEASUREMENT OF DOSE

When alpha or beta particles, or gamma radiation pass through matter, they form ions. They accomplish this by knocking electrons from the orbits of the molecules they pass through. We can monitor the ionization effect by allowing the radiation to pass through dry air and measuring the numbers of ions formed. This is most often done by designing a chamber with an electrical charge capacitance, allowing the radiation to pass through the chamber and monitoring the amount of capacitance discharge caused by the formation of ions. The device is a Geiger-Mueller Counter and has many variations.

The ionizing ability is measured in roentgens, and a roentgen is the number of ionizations necessary to form one electrostatic unit (e.s.u.) in 1 cc of dry air. Since the roentgen is a large unit, dosage for cell research use are normally divided into milliroentgens (mR).

Curies measure the amount of radioactive decay, roentgens measure the amount of radiation transmitted through matter, over distance. Neither unit is useful in determining biological effect, since biological effect implies that the radiation is absorbed by the tissues that are irradiated.

The rad (radiation absorbed dose) is a unit of absorbed dose and equals 100 ergs absorbed in one gram of matter. The roentgen is the amount of radiation exposure in air, while the rad represents the amount of radiation exposure in tissue. The two are usually very close in magnitude, however, since for most biological tissues, 1 roentgen produces 0.96 rad.

Not all radioactive emissions have the same penetrating power, however. If radiation safety (monitoring of dose) is considered, then the rad is insufficient. A linear-energy- transfer dependent factor must be defined for each type of emission. An alpha particle, for example, would not travel very far through tissue, but it is 10 times more likely to be absorbed than a gamma wave of the same energy dose. This factor is known as the Quality Factor (QF) or Relative Biological Effectiveness (RBE). The RBE is limited to work in radiobiology, the QF is used in other exposure monitor schemes. The use of the QF results in a new parameter, the rem. The rem is a unit of dose equivalent and is equal to the product of the QF x rad.

DETECTION OF RADIOACTIVITY

IONIZATION CHAMBERS The most common method of measuring radiation exposure is the use of an ionization chamber. Among the more common forms of ionization chambers are the Geiger counter and the pocket dosimeter.

The chambers are systems that comprise two electrical plates, with a potential established between them by a battery or other electrical source. In effect, they function as capacitors. The plates are separated by an inert gas, which will prevent any current flow between the plates. When an ionizing radiation enters the chamber, it induces the formation of an ion, which in turn is drawn to one of the electrical plates. The negative ions are drawn the the anode (+ plate) while the positive ions are drawn to the cathode (- plate). As the ions reach the plates, they induce an electric current to flow through the system attached to the plates. This is then expressed as a calibrated output,

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either through the use of a digital or analog meter, or as a series of clicks , by conversion of the current through a speaker.

The sensitivity of the system depends on the voltage applied between the electric plates. Since alpha particles are significantly easier to detect than beta particles, it requires lower voltage to detect the high energy alpha particles. In addition, alpha particles will penetrate through the metal casing of the counter tube, whereas beta particles can only pass through a quartz window on the tube. Consequently, ionization chambers are most useful for measuring alpha emmissions. High energy beta emissions are able to be measured if the tube is equipped with a thin quartz window and if the distance between the source of emission and the tube is minimal.

A modification of the basic ionization chamber can be made by engineering the tube such that it is miniaturized and such that the tube can be charged to hold a voltage without constantly rebuilding the voltage via a battery. This gives rise to the pocket dosimeter . This device is a capacitor, which is charged by a base unit and which can then be carried as a portable unit. They are often the size and shape of a pen and can be thus carried in the pocket of a lab coat. When exposed to an ionizing radiation source, the capacitor discharges slightly. Over a period of time, the charge remaining on the dosimeter can be monitored and used as a measure of radiation exposure. The dosimeters are usually inserted into a reading device which is calibrated to convert the average exposure the dosimeter has had directly into roentgens or rems. 1 Since the instrument works by discharging the built up charge, and the charge is upon a thin wire in the center of the dosimeter, it can be completely discharged by the flexing of that wire, as it touches the outer shell upon impact. When later read for exposure, the investigor will be informed that they have been exposed to dangerously high levels of radiation as there will be no charge left in the dosimeter. Besides causing great consternation with the Radiation Safety Officer, and a good deal of paper work, it also causes some unrest with the investigator. The dosimeters should be worn in a location where they can not impact any other objects.> Since the dosimeters normally lack the fragile and vulnerable quartz windows of a Geiger tube, and carry lower voltage potentials, they are used for the measurement of x- ray and high energy gamma radiation, and will not detect beta emmissions.

PHOTOGRAPHIC FILM Low energy emissions are detected more conveniently through the use of a film badge . This is simply a piece of photographic film sandwhiched between cardboard and made into a badge which can be pinned or clipped onto the outer clothing of the investigator. They can be worn routinely and collected on a regular basis for analysis.

When the film is exposed to radiation, it causes the conversion of the silver halide salts to reduced silver (exactly as exposure of the film to light). When the film is developed, the amount of reduced silver (black) can be measured and calibrated for average exposure to radiation. This is normally done by a lab specializing in the monitoring. Because of the simplicity of the system, its relative low cost and its sensitivity to nearly all forms of radiation, it is the primary means of radiation exposure monitoring of personnel.

SCINTILLATION COUNTERS For accurate quantitative measurement of low energy beta emissions and for rapid measurement of gamma emissions, nothing surpasses the use of scintillation counters. Since they can range from low to high energy detection, they are also useful for the alpha emissions.

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Scintillation counters are based on the use of light emitting substances, either in solution, or within a crystal. When a scintillant is placed in solution with a radioactive source (liquid scintillation counter), the radiation strikes the scintillant molecule, which will then fluoresce as it re-emits the energy. Thus the scintillant gives a flash of light for each radiation particle it encounters. The counter than converts light energy (either as counts of flashes, or as an integrated light intensity) to an electrical measure calibrated as either direct counts or counts per minute (CPM). If the efficiency of the system is known (the % of actual radiatioactive decays that result in a collision with a scintillant), then disintegrations per minute (DPM) can readily be calculated. DPM is an absolute value, whereas CPM is a function of the specific instrument used.

Low energy beta emissions can be detected with efficiencies of 40% or better with the inclusion of the scintillant directly into a cocktail solution. Alpha emissions can be detected with efficiencies in excess of 90%. Thus, with a liquid scintillation counter, very low doses of radiation can be detected. This makes it ideal for both sensitivity of detection and for safety.

If the system is modified such that the scintillant is a crystal placed outside of the sample chamber (vial) then the instrument becomes a gamma counter. Gamma emissions are capable of exiting the sample vial and entering into a fluorescent crystal. The light emitted from the crystal is then measured. Gamma counters are usually smaller than liquid scintillation counters, but are limited to use with gamma emittors. Modern scintillation counters usually combine the functional capabilities of both liquid scintillation and direct gamma counting.

Since all use of radioactive materials, and particularly the expensive counting devices are subject to local radiation safety regulations, the specific details for use must be left to the institutional discretion. Under no circumstances should radioactive materials be used without the express supervision of the radiation safety officer of the institution, following all specific institutional guidelines and manufacturer directions for the instrument used.

AUTORADIOGRAPHY

The process of localizing radioactive materials onto a cell is known as autoradiography. ^{3}H (tritium) is used in cell analysis because it is a relatively weak Beta emitter (thus making it safer to handle) and more significantly, can be localized within cell organelles. ^{14}C and ^{32}P are also used, but are more radioactive, require significantly more precautions in handling and are inherently less capable of resolving intracellular details. They are used at the tissue or organ level of analysis, however.

Radioactive isotopes can be incorporated into cellular molecules. After the cell is labeled with radioactive molecules, it can be placed in contact with photographic film. Ionizing radiations are emitted during radioactive decay and silver ions in the photographic emulsion become reduced to metallic silver grains. The silver grains not only serve as a means of detecting radioactivity but, because of their number and distribution, provide information regarding the amount and cellular distribution of radioactive label.

The process of producing this picture is therefore called autoradiography and the picture is called an autoradiogram.

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The number of silver grains produced depends on the type of photographic emulsion and the kind of ionizing particles emitted from the cell. Alpha particles produce straight, dense tracks a few micrometers in length. Gamma rays produce long random tracks of grains and are useless for autoradiograms. Beta particles or electrons produce single grains or tracks of grains. High energy Beta particles (such as those produced by 32P) may travel more than a millimeter before producing a grain. Low energy Beta particles (^{3}H and 14 ° C) produce silver grains within a few micrometers of the radioactive disintegration site and so provide very satisfactory resolution for autoradiography.

The site of synthesis of cellular molecules may be detected by feeding cells a radioactive precursor for a short period and then fixing the cells. During this pulse labeling, radioactivity is incorporated at the site of synthesis but does not have time to move from this site. The site of utilization of a particular molecule may be detected be chase labeling. Cells are exposed to a radioactive precursor, radioactivity is then washed or diluted away and the cells allowed to grow for a period of time. In this case, radioactivity is incorporated at the site of synthesis but then has time to move to a site of utilization in the cell.

^{3}H-thymidine can be used to locate sites of synthesis and utilization of DNA. Thymidine, the deoxyribose nucleoside of thymine, can be purchased with the tritium label attached to the methyl group of thymine. Thymidine is specifically incorporated into DNA in Tetrahymena. Some organisms can remove the methyl group from thymine, and incorporate the uracil product into RNA. Even in this case RNA would not be labeled because the tritium label would be removed with the methyl group. Methyl labeled thymidine, therefore, serves as a very specific label for DNA.

This is known as pulse labeling, after which the cells are washed free of the radioactive media. All remaining radioactivity would be due to the incorporation of the thymidine into the macromolecular structure of DNA. The cells will be fixed, covered with a photographic emulsion and allowed to develop .

During this time, the activity emanating from the ^{3}H will expose the photographic emulsion, causing the presence of reduced silver grains immediately above the location of the radioactive source (DNA). Thus, it will be possible to localize the newly synthesized DNA, or that which was in the S phase of mitosis during the time period of the pulse labeling.

RULES FOR SAFE HANDLING OF RADIOACTIVE ISOTOPES

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1. All work with radioactive material must be done in a tray lined with absorbant paper.
2. All glassware and equipment contacting radioactive material must be appropriately labeled and kept inside the tray. The only exception is that microscope slides of labeled cells may be removed from the tray after the drop of labeled cells has been applied to the slide and allowed to dry.
3. Plastic gloves should be worn when handling radioactive material.
4. All waste solutions containing radioisotopes, all contaminated gloves, paper, etc., must be placed in appropriate liquid or dry radioactive waste containers.

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- ALL INSTITUTIONAL REGULATIONS MUST BE FOLLOWED AT ALL TIMES.
- USE OF ANY RADIOACTIVE ELEMENT IS THE FULL RESPONSIBILITY OF THE INSTITUTION RADIATION SAFETY OFFICE AND ITS DESIGNATED OFFICERS.

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Appendix I: Photography

The use of photography within a cell biology laboratory allows for the capture of data and images for processing at a later time. It also is an excellent means of preparing materials for presentation, either through projection slides, or through illustrations.

PHOTOMICROGRAPHY

Photographically recording visual images observed through a light microscope is a useful means of obtaining a permanent record of activities. Using photomicrographs is the main means of recording electron microscope images.

Photomicrography begins with proper microscope use. It is important that the microscope be in good operation, clean and centered. Ideally, the microscope will be equipped with either a trinocular head, or a built in camera port. Excellent results can be had, however, by attaching a 35 mm single lens reflex camera to an eyepiece by way of an inexpensive adapter and a tripod or copy stand. The adapters are designed to fit in place of the lens, and the tripod is to take the weight of the camera off of the lens tube. The reflex camera is useful since it has its own focusing screen. Although you will not get a sharp image in the camera (because of the ground glass image plate), the negative will be sharp. Special camera attachments are available from the microscope manufacturers which incorporate excellent optical focusing devices, but these are costly. If a lot of photomicrography is to be performed, however, they are well worth the cost.

Use of a camera on the microscope is straightforward. Merely center the object to be photographed, focus using the camera viewer (that is, do not focus using the microscope eye piece) and depress the camera shutter button. Equipping the shutter with a shutter release cable will help prevent vibrations. This assumes that you have the proper exposure.

Exposure and film type are the major problems of photomicrography. For most microscopes using a tungsten lamp source, there is very little light reaching the camera. Film that has a high enough exposure index (ASA speed) are too grainy to be used for effective work. In general, the faster the film the less inherent resolution the film will have. As in all things in photography, a compromise is called for.

Moreover, most film sold for general use has a thicker film emulsion than is desirable. The microscope projects an image of very low contrast, with low light intensity. A thick emulsion tends to lower the contrast even more. This results in photographs that are all gray, with no highlights (black and white). The tonal range is reduced significantly using general film for photomicrography.

Use Kodak Technical Pan Film at an ASA of 100 for photomicrography. This is a thin emulsion film with extremely high contrast. The contrast can even be controlled through the developing process and ranges from High (used for photographing chromosomes), to moderate (used for general use) and low (not used in photomicrography). This same film can be used for copy work,

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since it reproduces images which are black and white. In general, you can not have too much contrast in a photomicrograph, but it is possible with this film.

Another means of increasing contrast is the use of colored filters within the microscope light path. Use a contrasting color to the object you wish to photograph. For example, chromosomes stained with aceto-orcein (dark red) can be contrast enhanced by the use of a green filter. Human chromosome spreads stained with Giemsa (blue) can be enhanced by the use of a red filter. This trick is also useful for routine viewing as well as photography.

The use of filters will increase the necessary exposure time. Technical Pan Film is also a relatively slow film. To establish the proper exposure, use the light meter built into the camera. If no light meter is available, you will have to shoot a roll of film and bracket several exposures to determine which is best. When using the built in meter, remember that all light meters are designed to give an image which is a medium gray. If you have a spot meter, be sure the spot is placed over an object which should be gray in the final image. If you have an averaging meter, be sure there is sufficient material in the viewfinder to give a proper average exposure. If you do not know whether you have a spot or averaging meter, find out. This is not trivial. Suppose you wish to photograph a chromosome spread. The chromosomes are typically less than 1-2% of the field of view. The meter will adjust the exposure such that the white field of light is exposed as gray, and your chromosomes will appear as darker gray on a gray field - in other words, extremely murky looking. Performing karyotype analysis on this type of image is difficult or impossible.

Bracket all exposures. Once you have determined the appropriate exposure, be sure to take several photographs. If, for example, the meter says 1/60 sec exposure, take another at 1/30 and one at 1/100. This process is known as bracketing the exposure to ensure that one is correct. Kodak Technical Pan Film is somewhat forgiving for poor exposure, but only somewhat. For black and white film, if you err on exposure, overexpose. This is the exact opposite for color positive film (slides).

For 35 mm cameras, be sure to rewind the film when all exposures have been completed.

PROCESSING

After exposure of the film, the film needs to be processed. Processing of black and white film has three steps. Develop the film, Stop it from developing, and Fix the emulsion so that it is no longer light sensitive.

You can send your film out for processing, but it will take longer, cost more, and in general you will be more pleased with your own work. The procedure requires about 30-40 minutes and can be done while cleaning up the lab.

PERFORM IN TOTAL DARKNESS:

Remove the film from the its cannister and roll it onto the developing tank reel. Place the film and reel into the developing tank and place the light prrof lid onto the tank. You may then turn on the lights. [1]

For processing, follow the following steps:

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1. Select the proper developer based on the film manufacter's recommendations. 2 Dilute the developer as recommended and measure the temperature.
   For Technical Pan film exposed at 100 ASA, use Kodak D-19 developer dilution D at a temperature of 21 ° C.
2. Pour the diluted developer into the tank and allow the solution to remain for the recommended time of development (6 20minutes for TP film). The development time is time and temperature dependent. Be sure to use the correct combination for the film used. During the development stage, gently swirl the solution in the tank once every 30 seconds. It also helps to invert the tank during this stage if the tank is equipped with a lid. If inverted, give the tank a mild rap on the bench top as you set it down. This will dislodge any trapped air bubbles from the film reel. Do not slam the tank down, it takes only a mild tap!
3. Stop the developing by pouring the developer from the tank and replacing it with Kodak Indicator Stop for 30 seconds.
4. Pour off the stop bath and replace it with Kodak Fixer for a period of 6-8 minutes. Kodak Rapid Fix may be substituted for a period of 2 minutes.
5. Pour off the fixer and replace with a 1:4 dilution of Kodak Hypo Clearing Agent for 2 minutes.
6. Pour off the clearing agent, open the tank and wash the film in running water for a period of no less than 5 minutes. After washing, rinse the film in distilled water (you may use Photoflo if available) and air dry the film.

PRINTING

Processing a roll of film results in a strip of negatives. For much of the photographic work in cell biology, it is not necessary to do anything with these negatives, except to store them, or use them directly for observation. Holding the negatives up to a light source, and viewing with a hand held magnifying lens is often sufficient. However, for presentation work, or for karyotype analysis, where you wish to cut out the chromosomes and rearrange them in some manner, a print needs to be made.

Printing the image is a more time consuming process than developing the film, requires a darkroom and is more expensive. As a minimum, there is a need for a photographic enlarger, developing trays and a good timer. Photographic paper is more expensive than film.

The basic process involves inserting the negative into the carrier of an enlarger, exposing a sheet of photographic paper to the projected negative and then processing the paper film in a manner similar to that for the film. The exception is that the Developer is switched to Dektol and the film is processed through trays rather than on a reel and within a tank. There are, however, many variations.

If many prints are to be made, a stabilization processor can be used. This works by virtue of the fact that the paper comes with the developer incorporated into the emulsion. It is activated by passing the paper through a bath of strong alkali, and halted by passing it through a strong acid. This is done automatically by a machine that resembles an old thermofax machine. The paper is fed into the front and rolls out as a photograph from the back. It is stable for about 6 months, unless fixed with a bath of Kodak Fixer, which will make it as permanent as any photograph. Electron microscopy labs will often have one of these instruments available.

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For details on printing (which has many aspects of an art form), refer to a text on photographic processing.

MACROPHOTOGRAPHY

Macrophotography is used to record things too large to be viewed in the microscope. This is an excellent means of making permanent records of electrophoresis gels, bands observed during ultracentrifugation, and whatever else you wish to capture on film.

Two changes are required from the use of photography through a microscope. The camera must be removed from the microscope and equipped with a lens, and secondly, the type of film used must be changed.

Very briefly, there are two means of adapting a 35 mm camera for macrophotography. The simplest is to purchase a macro lens. It is preferrable to use a real macro lens rather than a zoom with macro capability. Zoom lenses do not have the inherent resolution suitable for macro work. True macro lenses are, on the other hand, relatively expensive (~$300). An alternative solution is to purchase a lens reversal ring or a set of extension tubes for your camera. These are both inexpensive options. The former, use of a reversal ring, is an excellent means of optaining macro capability by simply turning around the normal 50 mm lens (cost about $10). The use of extension rings allows some variation in the magnification capabilities (~$30).

For occasional photos of electrophoresis gels, it is probably worth investing in a camera specifically designed for that purpose. Fotodyne markets a Polaroid camera gun for this purpose (~$600). At the other extreme is the use of an MP4 Polaroid copy stand ($2,000) for macrophotography. The most economical means of performing routine photography is to purchase a used 1960's Pentax Spotmatic (or equivalent) with lens reversal ring and a microscope adapter. Combined with a bulk film loader and a changing bag, nearly all the required aspects of film recording of data can be readily accomplished at bargain prices.

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Appendix J: Chemical Preparations

The following is a list of the solutions and chemicals required throughout the laboratory manual. It is organized alphabetically, and individual exercises list the materials needed for that exercise. For many solutions, directions are given for a molar solution and the user is left to dilute to the appropriat e concentration for their needs. There are many vendors of the chemicals listed, and many of the solutions can also be purchased pre-mixed.

Acetic acid (MW 60.05)

Glacial acetic acid is 99.6% (w/v) acetic acid, and is 17.4 M.

1 M	Add 57.5 ml of glacial acetic acid to 800 ml of water and then make to 1 liter with water.
0.05 N	Add 2.87 ml of glacial acetic acid to 800 ml of water and then make to 1 liter with water.
0.9 M	Add 54 ml of glacial acetic acid to 800 ml of water and then make to 1 liter with water.
7% (w/v)	Add 70 ml of glacial acetic acid to 800 ml of water and then make to 1 liter with water.
45% (w/v)	Add 450 ml of glacial acetic acid to 500 ml of water and then make to 1 liter with water.

Acetic Acid/Butanol/Water (15:60:25)

Combine 150 ml of glacial acetic acid, 600 ml of nbutanol and 250 ml of water.

Aceto-orcein

Add 2.0 grams of orcein to 45 ml of glacial acetic acid. Bring to a boil and continue to heat until completely dissolved. Cool and add 55 ml of distilled water. Filter prior to use. Note: Some early investigators added a of an iron salt (such as ferric citrate) as a mordant. It tends to increase the intensity of the aceto-orcein stain. The same reaction can be had by chopping plant material with an older steel (not stainless) razor blade.

Acid alcohol

Add 1.0 ml of concentrated HCl to 100 ml of 70% (v/v) ethyl alcohol.

Acid Orcinol Reagent (0.1% $FeCl_3$ in 10% HCl)

Add 0.1 grams of $FeCl_3$ to 50 ml of 10% (v/v) HCl and make to 100 ml with 10% HCl.

Acrylamide Solutions

Acrylamide solutions for PAGE are given as total concentration of acrylamide (acrylamide + bisacrylamide) and the amount of cross linker (bisacrylamide). This is listed as the T:C ratio. For example, a 10% gel (10%T:5%C) would contain a total of 10 g rams of acrylamide per 100 ml, and would be composed of 5 grams of acrylamide and 5 grams of bisacrylamide. Usually, a stock solution of 30% acrylamide is produced containing 0.8% bis-acrylamide. Many investigators use 30 grams of acrylamide plus 0.8 gram s of bis-acrylamide per 100 ml of water, but 29.2 grams of acrylamide plus 0.8 grams of bis would be technically correct. In practice, it makes little difference since the gels are diluted to 10% or less. The 30% stock solution is filtered through a 0.45 µ filter and stored at 4 ° C in the dark. For use, the stock solution is diluted with an appropriate buffer (usually a 2X Tris-HCl). The stock solution is stable for about one month. Discard after this period.

Acrylamides in their monomeric form are neurotoxic. Polymerize all acrylamide solutions prior to

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disposal.

SDS, β-mercaptoethanol and a tracker dye (bromophenol blue) are added at various points. Refer to Chapter 4 for more complete details.

Alcian blue (MW ~1300)

0.001 M	Dissolve 0.13 grams of Alcian Blue 8GX (Sigma # A-2899) in 100 ml of water.

Alcohol Orcinol Reagent (10% orcinol in 95% ethanol)

Dissolve 1.0 gram of orcinol in 95% ethanol to a final volume of 10 ml .

Alkaline Distilled Water

Add one pellet of NaOH to 1 liter of distilled water.

Alkaline Solution for G-banding

Dissolve 2.8 grams of NaOH and 6.2 grams of NaCl to a final volume of 1 liter with water.

p-Aminosalicic Acid (PAS MW 175.1)

6% (w/v)	Dissolve 6.0 grams of PAS to a final volume of 100 ml with water or buffer.

Ammonium acetate (MW 77.08)

0.1 M	Add 7.708 grams of Ammonium acetate to a final volume of 1 liter of water.

Ammonium persulfate (MW 228.2)

10% (w/v)	Dissolve 1.0 grams of ammonium persulfate to a final volume of 10 ml with water. Mix fresh, prior to use as a catalyst for PAGE. Normally, about 50 µ l of ammonium persulfate is added to each 15 of gel solution for polymerization.Dissolve 0.13 grams o f Alcian Blue 8GX (Sigma # A-2899) in 100 ml of water.

Ammonium sulfate (MW 132.14)

2% (w/v)	Add 2 grams of ammonium sulfate to a final volume of 100 ml water.
4.1M(sat.) 0.001 M	Dissolve 542 grams of ammonium sulfate to a final volume of 1 liter.

n-Amyl alcohol (Pentanol $C_5H_{11}OH$ MW 88.15)

Density = 0.8144 grams/ml

0.38 M	The amyl alcohol can be weighed (33.5 grams) or measured volumetrically by using the density. That is, 33.5 grams ÷0.8144 grams/ml or 41.1 ml of n-amyl alcohol. Weigh or measure the appropriate amount and dilute to a final volume of 1 liter with water.

Amylase, buffered pH 7.0

1% (w/v)	Dissolve 0.5 grams of amylase to a final volume of 50 ml with 0.01 M sodium phosphate buffer, pH 7.0.

Ascorbic acid (MW 176.12)

2 mM	Dissolve 35.2 mg of ascorbic acid to a final volume of 100 ml with water.

ATP (Adenosine triphosphate, MW 507.21)

5 mM	Dissolve 254 mg of ATP to a final volume of 100 ml with water or buffer.Dissolve 35.2 mg of ascorbic acid to a final volume of 100 ml with water.

Baker's Formalin

Add 1.0 gram of calcium chloride, 1.0 gram of cadmium chloride and 10 ml of concentrated formalin to

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75 ml of water. Make to a final volume of 100 ml with water.

Benzoic acid (MW 122.12)

8 mM	Dissolve 98 mg of benzoic acid to a final volume of 100 ml with water or buffer.

Bis-acrylamide (N,N'-Methylene-bis-acrylamide)

Cross linker for acrylamide gels. Refer to Acrylamide solutions or Chapter Four for more details.

Biuret Reagent

Add 1.50 grams of $CuSO_4 \cdot H_2O$ and 6.0 grams of sodium potassium tartrate to 500 ml of water. Separately make 300 ml of 10% (w/v) NaO H by dissolving 300 grams of NaOH to a final volume of 300 ml with water. Combine the two solutions in a 1 liter volumetric, swirl to mix and make up to 1 liter with water. Store the final solution in a dark, plastic bottle. Discard if black or red precip itate forms.

Bovine Serum Albumin (BSA)

There are many grades of BSA available and care should be taken when using this protein. For routine protein concentration standards, a 96-99% pure fraction (Sigma # A 2153) may be used. For tissue culture, RIA, or molecular weight standardization, BSA sh ould be optained which is extracted and purified specifically for that purpose.

1% (w/v)	Dissolve 0.5 grams of BSA to a final volume of 50 ml in water or buffer.

Bradford Protein Assay

This procedure uses an absorbance shift in an acidic Coomasie Blue solution. It is commercially available from Pierce Chemical Company, Rockford, Illinois as Protein Assay Reagent, Cat. # 23200. It contains methanol and solubilizing agents and is very rel iable.

If you wish to make your own, dissolve 100 mg of Coomasie Brilliant Blue G-250 in 50 ml of 95% ethanol. Add 100 ml of 85% phosphoric acid, and bring to a final volume of 1 liter with distilled water.

Phosphoric acid is extremely corrosive. Handle with care.

Bromophenol blue (Sodium salt, MW 692.0)

0.001 (w/v)	Dissolve 1 mg of Bromophenol blue, sodium salt (Sigma # B7021) to a final volume of 100 ml with either water or buffer.

n-Butanol (C_4H_9OH MW 74.12)

Density = 0.8098 grams/ml

1.1 M	The butanol can be weighed (81.5 grams) or measured volumetrically by using the density. That is, 81.5 grams ÷0.8098 grams/ml or 100.7 ml of n-butanol. Weigh or measure the appropriate amount and dilute to a final volume of 1 liter with water.

C. elegans Ringers

This is a basic saline solution for nematodes. Dissolve 11.36 grams of $Na_2HPO_4 \cdot 7H_2O$, 3.0 grams of KH_2PO_4, 0.5 grams of NaCl and 1.0 gram of NH_4Cl to a final volume of 1 liter. Adjust the pH to 7.0. May be autoclaved for sterilization.

Calcium chloride (MW 110.99)

0.0033 M	Dissolve 0.522 grams of calcium acetate to a final volume of 1 liter with water or buffer.

Calcium chloride (MW 110.99)

0.001 M	Dissolve 0.111 grams of anhydrous calcium chloride to a final volume of 1 liter with water or

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	buffer.
0.08 M	Dissolve 8.879 grams of anhydrous calcium chloride to a final volume of 1 liter with water or buffer.
2% (w/v)	Dissolve 2 grams of anhydrous calcium chloride to a final volume of 100 ml with water or buffer.

cAMP (Adenosine monophosphate, cyclic MW 329.22)

0.001 M (1mM)	Dissolve 33 mg of cAMP to a final volume of 100 ml with water, buffer or media.

Carnoy fixative

Combine 10.0 ml of glacial acetic acid with 60.0 ml of absolute ethyl alcohol and 30.0 ml of chloroform.

Chloroplast homogenization buffer

To 400 ml of distilled water, add 30.058 grams of sorbitol, 2.23 grams of sodium pyrophosphate, 0.407 grams of magnesium chloride, and 0.176 grams of ascorbic acid. Adjust the pH to 6.5 with HCl and dilute to a final volume of 500 ml.

Chloroplast suspension buffer

To 400 ml of distilled water, add 30.058 grams of sorbitol, 0.372 grams of EDTA, 0.102 grams of magnesium chloride and 5.958 grams of HEPES buffer. Adjust the pH to 7.6 with NaOH and dilute to a final volume of 500 ml.

Chrom alum gelatin (Subbing solution)

Dissolve 5.0 gram of gelatin in 1 liter of boiling water. Cool and add 0.5 grams of potassium chrome alum ($CrK(SO_4)_2 \cdot 12H_2O$). Store in refrigerator.

To use, dip clean slides into the solution and dry in vertical position in a dust free location.

Citric acid ($H_3C_6H_5O_7 \cdot H_2O$ MW 210.14)

0.1 M	Dissolve 21.01 grams of citric acid to a final volume of 1 liter.

Citrate buffer (Sodium phosphate-Citrate buffer)

0.001 M	
PH 4.8	Add 493 ml of 0.2 M Na_2HPO_4 to 507 ml of 0.1 M citric acid.
PH 3.6	Add 322 ml of 0.2 M Na_2HPO_4 to 678 ml of 0.1 M citric acid.
PH 4.2	Add 414 ml of 0.2 M Na_2HPO_4 to 586 ml of 0.1 M citric acid.
PH 5.4	Add 557.5 ml of 0.2 M Na_2HPO_4 to 442.6 ml of 0.1 M citric acid.
PH 6.0	Add 631.5 ml of 0.2 M Na_2HPO_4 to 368.5 ml of 0.1 M citric acid.
PH 6.6	Add 727.5 ml of 0.2 M Na_2HPO_4 to 272.5 ml of 0.1 M citric acid.
PH 7.2	Add 869.5 ml of 0.2 M Na_2HPO_4 to 130.5 ml of 0.1 M citric acid.
PH 7.8	Add 957.5 ml of 0.2 M Na_2HPO_4 to 42.5 ml of 0.1 M citric acid.

Cobaltous Nitrate (MW 182.96)

2% (w/v)	Dissolve 2.0 grams of cobaltous nitrate (hexahydrate is very soluble) to a final volume of 100 ml with water. Keep well closed in a cool place.

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Colcemid

10 µ g/ml	Dissolve 10 g of colcemid per ml of saline or culture medium.

Coomasie blue (Coomasie Brilliant Blue R250)

0.25% (w/v) 0.001 M	Dissolve 2.50 grams of Coomasie Brilliant Blue R250 to a final volume of 1 liter with 20% (w/v) trichloroacetic acid (TCA). Some investigators use a 0.25% solution of Coomasie Blue in methanol-water-glacial acetic acid (5-5-1).

Copper sulfate ($CuSO_4 \cdot 5H_2O$ MW 249.68)

0.5% (w/v)	Dissolve 0.13 grams of Alcian Blue 8GX (Sigma # A-2899) in 100 ml of water.

0.5% (w/v)

Dissolve 0.5 grams of copper sulfate to a final volume of 100 ml with water.

Copper tartrate/carbonate (CTC)

Dissolve 0.5 grams of copper sulfate and 1.0 gram of potassium sodium tartrate to a final volume of 100 ml with water. Combine 1.0 ml of this solution with 50 ml of 2% Na_2CO_3 in 0.1 N NaOH. Must be made fresh, prior to use. Stock solutions are stable.

Crystal Violet

Dissolve 0.1 grams of crystal violet and 0.25 ml of glacial acetic acid to a final volume of 100 ml with water.

DCMU (3-(3,4-Dichlorophenyl)-1,1-Dimethylurea MW 233.1)

1 x 10^{-4} M 0.001 M	Dissolve 2.3 mg DCMU to a final volume of 100 ml with water or buffer.
5 x 10^{-7} M0.001 M	Dilute the 1 x 10^{-4} M solution 1/200 prior to use.

Dichlorophenolindophenol (DCPIP MW 290.1)

0.0025 M	Dissolve 73 mg of DCPIP to a final volume of 100 ml with water or buffer.
0.0001 M	Dissolve 2.9 mg of DCPIP to a final volume of 100 ml with water or buffer.

Dinitrophenol (DNP MW 184.11)

18.4 mg%	Dissolve 18.4 mg of 2,4-dinitrophenol to a final volume of 100 ml with water or buffer.

Dische diphenylamine reagent

Dissolve 500 mg of diphenylamine in 49 ml of glacial acetic acid. Add 1.0 ml of concentrated HCl.

Dithiothreitol (Cleland's Reagent MW 154.3)

0.01 M	Dissolve 154 mg of dithiothreitol to a final volume of 100 ml with water or buffer. Dithiothreitol is available from Sigma Chemical Co., St. Louis, Cat # D0632. Dithioerythritol may be substituted.

DOPA (3-(3,4-Dihydroxyphenyl)-L-alanine MW 197.19)

8 mM	Dissolve 158 mg of L-DOPA to a final volume of 100 ml with water or buffer. Note that the maximum solubility of DOPA in water is 165 mg/100 ml (8.3 mM).

EDTA (Ethylenediaminetetraacetic acid MW 292.24)

1 M	Dissolve 292.24 grams of EDTA, free acid to a final volume of 1 liter. If the more soluble disodium salt of EDTA is used, adjust the weight accordingly. The pH can be adjusted with acetic acid or NaOH. For corresponding concentration dilutions, multiply the weight in grams by

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	the desired molarity. For exam ple, for 10 mM EDTA, multiply 292.24 X 0.010 to obtain 2.92 grams of EDTA per liter.

EGTA (Ethylene Glycol-bis(β-aminoethyl Ether) N,N,N',N'-Tetraacetic Acid MW 380.4)

1 mM	issolve 380 mg of EGTA to a final volume of 1 liter with water or buffer.

Eosin

0.5% (w/v)	Dissolve 0.5 grams of Eosin Y in 100 ml of water.

Ethanol (C_2H_5OH MW 46.07)

Density = 0.7893 gm/ml

50-95% (v/v)	Since 95% ethyl alcohol is less expensive and easier to store than absolute, these dilutions should be made with 95% ethyl alcohol. Unless otherwise stated, denatured alcohol works as well as the more expensive non-denatured. A simple way to make the % so lution is to use the appropriate amount of 95% ethanol and dilute to 950 ml instead of 1 liter. For example, to make a 50% (v/v) solution, measure out 500 ml of 95% ethyl alcohol and dilute to a final volume of 950 ml with water. For a 70% solution, meas ure 700 ml of ethyl alcohol and dilute to 950 ml with water. Absolute ethanol should be used directly as 100% ethanol. It is important for histology that this be truly 100%. Since it is hydroscopic (it absorbs water from the air), do not assume it is abso lute unless it is sealed or treated to ensure no water. To test, add a drop to a sample of xylol. If any cloudiness occurs, the alcohol is not absolute.
8.5 M	The ethanol can be weighed (391.6 grams of absolute, 412.2 grams of 95% (v/v) or measured volumetrically by using the density. That is, 391.6 grams ÷0.7893 grams/ml or 496.1 ml of absolute ethanol. Using 95%, 412.2 grams ÷0.7893 grams /ml or 522.2 ml. Weigh or measure the appropriate amount and dilute to a final volume of 1 liter with water.

Ethanol-acetic acid fixative for histology (3:1)

To 75 ml of absolute alcohol, add 25 ml of glacial acetic acid. Must be made fresh, just prior to use.

Fetal Calf Serum (FCS)

While it is possible to prepare your own serum from whole blood, it is easier (and safer) to purchase FCS from a reputable supplier. Commercial sources are free of mycoplasma, pre-sterilized and controlled for the presence of antibodies. There are a n umber of serum substitutes available on the market and these may be less expensive when storage is considered. Suppliers include Gibco, Flow Laboratories, KC Biological and Sigma Chemical Co.

Folin-Ciocalteu Reagent

This is usually purchased premixed, since it is difficult to make. Also known as 2N Folin-phenol reagent.

Giemsa stain

Prepare a stock solution by dissolving 3.8 grams of giemsa powder in 25 ml of glycerin. Heat gently with stirring for about 2 hours at 60 ° C. Cool and add 75 ml of methanol (neutral, acetone free).

For a working solution, dilute the stock solution 1/10 with water before use.

For chromosome banding, combine 5.0 ml of stock Giemsa, 3.0 ml of absolute methanol, 3.0 ml of 0.1 M citric acid and 89 ml of distilled water. Adjust the pH of the solution to 6.6 with Na_2HPO_4.

Glucose (MW 180.16)

10% (v/v)	Dissolve 10 grams of D-glucose (dextrose) in a final volume of 100 ml with water or buffer.

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Glutaraldehyde (GTA)

5 %	GTA is usually supplied as a 25% or 50% (w/v) solution. It is used for electron microscope fixation as a 5% solution in a buffer. For routine use, add 20 ml of 25% GTA to 80 ml of 0.2 M sodium cacodylate buffer, pH 7.4.

Glycerol (MW 92.09)

10% (v/v)	To 10 ml of glycerol (glycerine) add enough water to make a final volume of 100 ml.
8 M	Weigh 73.67 grams of glycerol and add to a final volume of 100 ml. Alternatively, measure 499.1 ml of glycerol and make to a final volume of 1 liter (the density of glycerol at room temperature is 1.476) with water or buffer. For 8 M glycerol in MT buffer, make a 2X MT buffer for use as the diluent.

Glycine (MW 75.07)

0.192 M	Dissolve 1.44 grams of Glycine to a final volume of 100 ml with water or buffer.

Gram's iodine

Dissolve 0.33 grams of iodine and 0.67 grams of potassium iodide to a final volume of 100 ml with water.

HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] MW 238.3)

50 mM	Dissolve 11.92 grams of HEPES, free acid to a final volume of 1 liter. If hemisodium salt is used, adjust weight accordingly. Do not use sodium salts unless specified. Hemisodium salt contains 0.5 moles of sodium for each mole of HEPES.
10 mM pH7.6	Dissolve 2.38 grams of HEPES, free acid in 900 ml of water. Adjust the pH with NaOH or HCl to 7.6. Adjust the final volume to 1 liter with distilled water.

Hydrochloric Acid (HCl MW 36.46)

Concentrated HCl has a molarity of approximately 11.6.

HCl is a gas, which is soluble in water and which comes in the form of concentrated reagent grade HCl. This solution is approximately 36-38% (w/v) HCl. To make a 1 N solution, add 86 ml of concentrated HCl to 800 ml of water and dilute to a final volume of 1 liter. For 0.1 N, dilute the 1 N by a factor of 10.

For % solutions, note that liquid HCl is only 38% HCl, thus a 1% solution would require 2.6 ml of concentrated HCl (1/.38) per final volume of 100 ml.

Janus Green B

0.01%(w/v)	Dissolve 10 mg of Janus Green B in 2-3 ml of absolute ethanol. Dilute to a final volume of 100 ml with water.

Knudson Media

Knudson X4 Stock:	
$Ca(NO_3)_2 \cdot 4H_2O$	4.0 grams
$(NH_2)SO_4$	2.0 grams
$MgSO_4 \cdot 7H_2O$	1.0 gram
Distilled H_2O	1.0 liter

B5 Minor Elements:

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H_2SO_4	0.5 ml
$MnCl_2 \cdot 4H_2O$	2.5 grams
H_3BO_3	2.0 grams
$ZnSO_4 \cdot 7H_2O$	50 mg
$CoCl_2 \cdot 6H_2O$	30 mg
$CuCl_2 \cdot 2H_2O$	15 mg
$Na_2MoO_4 \cdot 2H_2O$	25 mg
Distilled H_2O	1.0 liter

Ferric Citrate:

$FeC_6H_5O_7 \cdot 5H_2O$	2.5 grams
Distilled H_2O	100 ml

Stock Phosphate:

K_2HPO_4	25 grams
Distilled H_2O	100 ml

1X Media	Add 250 ml of Knudson X4 to 750 ml of distilled water. Add 0.5 ml of B5 Minor Elements, 0.5 ml of Stock Phosphate, and 0.4 ml of Ferric citrate. Adjust the pH to 5.5 with HCl, add 15 grams of agar and heat to dissolve. Autoclave and pour into plates.< br>
Note:	2.50 grams of sucrose may be added prior to adjustment of the pH, if desired. It is not necessary for germination of spores, but adds an organic source for mutants and abnormal fern growths. It also increases the need for subsequent aseptic technique .

Krebs Phosphate Ringers (KPR)

Prepare each of the following separately:

0.90% (w/v) NaCl
1.15% (w/v) KCl
1.22% (w/v) $CaCl_2$
3.82% (w/v) $MgSO_4 \cdot H_2O$
0.1 M phosphate buffer, pH 7.4 (17.8 grams of $Na_2HPO_4 \cdot H_2O$
+ 20 ml of 1 N HCl, diluted to 1 liter)

To mix, combine 200 ml of NaCl, 8 ml of KCl, 6 ml of $CaCl_2$ and 2 ml of $MgSO_4$. Carefully, and with constant stirring, add 40 ml of phospha te buffer.

LPS buffer (Lower Pad Solution buffer)

Dissolve 1.5 grams of KCl, 0.5 grams of $MgCl_2$, and 0.5 grams of steptomycin sulfate in 500 ml of water. Add 40 ml of 1 M phosphate buffer, pH 6.5 and dilute to 1 liter with water.

Magnesium chloride ($MgCl_2$ MW 95.23)

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1 mM	Dissolve 95.2 mg of magnesium chloride per final volume of 1 liter.
4 mM	Dissolve 0.381 grams of magnesium chloride per final volume of 1 liter.
10 mM	Dissolve 0.952 grams of magnesium chloride per final volume of 1 liter.
0.1 M	Dissolve 9.523 grams of magnesium chloride per final volume of 1 liter.
Note:	A single stock solution of 1 M $MgCl_2$ can be mixed by dissolving 95.23 grams of magnesium chloride to a final concentration of 1 liter with water, and all dilutions made appropriately from this stock solution.

Magnesium sulfate ($MgSO_4$ MW 120.39)

5% (w/v)	Dissolve 5.0 grams of magnesium sulfate to a final volume of 100 ml with water or buffer.

Mayer's hematoxylin

Purchase commercially or mix with either of the following procedures:

A. Dissolve 1.0 gram of hematoxylin in 10 ml of absolute ethanol. Dissolve 20 grams of potassium alum ($KAl(SO_4)_2 \cdot 12H_2O$) in 200 ml of water. In a chemical hood, with protection against explosion, bring the potassium alum solution to a boil and add hematoxylin/ethanol mixture. Continue to boil for approximately 1 minute. Add 0. 5 grams of mercuric oxide and cool rapidly. Add 0.5 ml of glacial acetic acid. Filter before use. This mixture is stable for about two months.

B. Alternatively: Dissolve 5.0 grams of hematoxylin in 50 ml of absolute ethanol and add to 650 ml of warm water. Heat gently until the hematoxylin dissolves and then add 300 ml of glycerin, 0.3 grams of sodium iodate and 20 ml. of glacial acetic acid. Co ol and make volume up to 1 liter with distilled water. Filter before use.

<DTβ>-Mercaptoethanol (MW 78.13)

0.5 M	Density = 1.2 grams/ml. Use either 3.91 grams OR 3.26 ml of mercaptoethanol in a final volume of 100 ml of water or buffer.
5% (w/v)	Use 5.0 grams or 4.167 ml in a final volume of 100 ml of water or buffer.

MES (2-(N-Morphilino)ethanesulfonic acid MW 195.2)

0.1 M	Dissolve 1.952 grams of MES to a final volume of 100 ml with water or buffer.

Methanol (CH_3OH MW 32.04)

Density = 0.7914 grams/ml	
22 M	The methanol can be weighed (704.9 grams) or measured volumetrically by using the density. That is, 704.9 grams ÷0.7914 grams/ml or 890.7 ml of methyl alcohol. Weigh or measure the appropriate amount and dilute to a final volume of 1 liter with water.

Methanol/Acetic Acid (for fixing proteins in acrylamide gels)

45%:12%	Add 120 ml of glacial acetic acid to 450 ml of methanol and dilute to a final volume of 1

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	liter with water.

Methanol/Acetic Acid (for destaining or fixing proteins in acrylamide gels)

5%:7%	Add 70 ml of glacial acetic acid to 50 ml of methanol and dilute to a final volume of 1 liter with water.

Methyl green

0.2% (w/v)	Dissolve 0.2 grams of methyl green to a final volume of 100 ml with 0.1 M acetate buffer, pH 4.2. Acetate buffer (0.1 M pH 4.2) is prepared by dissolving .361 grams of sodium acetate (trihydrate) in approximately 80 ml of water. Add .42 ml of glacial acetic acid and adjust the volume to 100 ml with water.

Microtubule buffer (MT buffer)

Dissolve 19.52 grams of MES in 800 ml of distilled water. Add 0.380 grams of EGTA and 47.62 grams of $MgCl_2$. Adjust the pH to 6.4 with HCl or NaOH and dilute to a final volume of 1 liter with disti lled water.

Minimum essential medium (MEM)

For all purposes of this manual, MEM refers to Eagle's MEM. While it is possible to mix this medium, it is infinitely easier (and less expensive) to purchase the media pre-mixed from any number of commercial sources (Gibco, Flow, KC Biological, Sigma Chem ical). The ingredients are listed in Table 12.1. It is essential that chemicals of the highest purity are used throughout.

NG agar (Nematode Growth agar)

Dissolve 3.0 grams of NaCl, 2.5 grams of peptone, and 17 grams of agar in a final volume of 1 liter. Boil to dissolve the agar, autoclave to sterilize.

Meanwhile, prepare separate sterile solutions of:

1 M $CaCl_2$
2 mg/ml uracil
10 mg/ml cholesterol in ethanol
1 M Potassium phosphate buffer, pH 6.0
1 M $MgSO_4$

Using proper sterile technique, cool the agar solution slightly and add 1 ml of $CaCl_2$, 1 ml of uracil, 0.5 ml of cholesterol, 25 ml of phosphate buffer, and 1 ml of $MgSO_4$. Swirl to mix all ingredients and pour plates.

p-Nitrophenyl phosphate (MW 263.1)

0.05 M	Dissolve 1.32 grams of -nitrophenyl phosphate to a final volume of 100 ml with water or buffer.
0.8% (w/v)	Dissolve 0.8 grams of -nitrophenyl phosphate to a final volume of 100 ml of water or buffer.
Note:	Sigma Chemical Co., St. Louis, supplies this compound as Sigma 104, Phosphatase Substrate, Cat. # 104-0.

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Osmium tetroxide (OSO_4 MW 254.2)

1%	Osmium tetroxide is a gas which is used in solution for EM preservation. It is best purchased in sealed vials of 2 ml of 4% OsO/4/. For use, add 6.0 ml of water or buffer to the 2.0 ml of 4% osmium tetroxide. Seal in a tightly sealed container, wrapped with aluminium foil and keep in the refrigerator. Use of a fume hood is mandatory when using OsO/4/. Osmium tetroxide will rapidly fix the nasal passages and exposed cornea if not properly vented. It should be handled with extreme care.

Perchloric acid (PCA MW 100.47)

2% (w/v)	Dissolve 2.0 grams of PCA to a final volume of 100 ml with water or buffer.

Percoll

Colloidal PVP coated silica. Available from Sigma Chemical Co., St. Louis. Cat. #P 1644.

Periodic Acid (Periodate Used for PAS reaction)

Dissolve 0.6 grams of periodic acid in 100 ml of water and add 0.3 ml of concentrated nitric acid.

Phenazine methosulfate (PMS MW 306.34)

Mutagen and irritant.

0.033% (v/v)	Add 33 µ l of phenazine methosulfate to 90 ml of water or buffer and make up to 100 ml final volume. Must be made immediately prior to use.

Phenol mixture

Combine 555 ml of aqueous phenol (or 500 grams of phenol crystals plus 55 ml of water) with 70 ml of mcresol. Add 0.5 grams of 8-hydroxyquinoline.

Phenol will cause severe burns and readily dissolves all plastic and rubber compounds. Use extreme caution when handling this compound.

P-Phenylenediamene oxalate (PPDO MW 198.18)

0.02% (w/v)	Dissolve 20 mg of PPDO to a final volume of 100 ml with water or buffer.

Phosphate buffered saline (PBS)

Mix 100 ml Ca^{++}/Mg^{++} fr ee 10X PBSA with 800 ml of distilled water. Separately, dissolve 0.1 gram of magnesium chloride and 0.1 gram of anhydrous calcium chloride to a final volume of 100 ml with water. With constant stirring, slowly add the magnesium/calcium chloride solution t o the diluted PBSA. If a precipitate forms, start over, and add slower with continuous stirring.

Ca^{++}/Mg^{++} free Phos phate buffered saline - 10X (10X PBSA)

Dissolve 80 grams of NaCl, 2.0 grams of KCl, 15.0 grams of Dibasic sodium phosphate and 2.0 grams of Monobasic potassium phosphate in 1 liter of distilled water. This makes a 10X solution of Ca^{++}/Mg^{++} *free phosphate buffered saline. Dilute 1:10 prior to use. Store in a refrige rator.*

Phosphate buffered saline-Tween 20 (4.8)

Mix PBS and add 0.1% (v/v) Tween 20.

Phytohemaglutinin (PHA)

Available as kidney bean lectin. It is typically used as a stock solution of 10-20 g/ml in balanced salt solution. For tissue culture it must be cold sterilized prior to use.

Potassium chloride (KCl MW 74.55)

1 M	Dissolve 74.55 grams of KCl to a final volume of 1 liter with water or buffer. For other

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	concentrations, multiply the weight by the required molarity. For example, for 0.150 M (150 mM), use 0.150 X 74.55, or 11.183 grams of KCl in 1 liter of water or buffer. Use half as much to obtain 0.075 M for karyotyping.

Potassium cyanide (KCN MW 65.11)

8 mM	Dissolve 52 mg KCN to a final volume of 100 ml with water or buffer.

Potassium phosphate, monobasic (KH_2PO_4 MW 136.09)

0.01M	Dissolve 1.36 grams of monobasic potassium phosphate to a final volume of 1 liter with water.

Potassium phosphate, dibasic (K_2HPO_4 MW 174)

0.01 M	Dissolve 1.74 grams of dibasic potassium phosphate to a final volume of 1 liter with water.

Potassium phosphate buffer

0.01M pH 7.4	Prepare 500 ml of 0.01 M K_2HPO_4 and 500 ml of 0.01 M KH_2PO_4 Place the K_2HPO_4 onto a magnetic stirrer and insert a pH electrode. Ad d the KH_2PO_4 slowly to adjust the pH to 7.4.

Potassium hydroxide (KOH MW 56.10)

0.5 N	Dissolve 28.05 grams of KOH to a final volume of 1 liter with water.
10% (w/v)	Dissolve 10 grams of KOH to a final volume of 100 ml with water. Store in a plastic container.

Potassium sodium tartrate (Rochelle salt $KNaC_4H_6O_6 \cdot 4H_2O$ MW 282.23)

n-Propanol (C_3H_7OH MW 60.11)

Density = 0.8035 grams/ml

3 M	The n-propanol can be weighed (180.3 grams) or measured volumetrically by using the density. That is, 180.3 grams ÷0.8035 grams/ml or 224.4 ml of n-propanol. Weigh or measure the appropriate amount and dilute to a final volume of 1 liter with water.

Protein Buffer

Dissolve 1.46 grams of KH_2PO_4 and 0.92 grams of $K2HPO_4$ in 80 ml of distilled water. Add 2.5 grams of crystalline serum albumin and adjust the volume to a final 100 ml with water.

Pyronin Y (acetone)

0.6% (w/v)	Dissolve 0.6 grams of pyronin Y in 100 ml of acetone.

Ribonuclease

0.1% (w/v)	Dissolve 10 mg of pancreatic ribonuclease type A in 10 ml of water or buffer. Use for enzyme treatment of histological sections by floating 0.5-1.0 ml of this solution onto the section, with the slide set into a covered petri plate.

Safranin

Dissolve 2.5 grams of Safranin O in 10 ml of 95% ethanol and dilute to 100 ml with water.

Saline (NaCl)

0.85% (w/v)	Saline refers to a solution of NaCl, with the most common usage for that which is isotonic to mammalian blood cells, notable a 0.85% or 0.9% solution. To mix, dissolve 8.5 grams of NaCl to a final volume of 1 liter with water.

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Saline citrate (1/10 dilution of SSC)

Dissolve 0.878 grams of NaCl and 0.294 grams of sodium citrate to a final volume of 1 liter with water.

Saline citrate buffer (SSC)

20X	It is common to prepare this buffer as a stock 20X solution, to be diluted to 2X, 1X or 0.1X prior to use. To prepare a 20X stock solution, dissolve 175 grams of NaCl and 88 grams of sodium citrate in 900 ml of water. Adjust the pH to 7.0 with 1 N HCl and bring to a final volume of 1 liter. For use, as a 1X SSC, dilute 1 part 20X stock with 19 parts distilled water. For a 2X SSC, dilute 1 part 20X stock with 9 parts water.

Schiff's Reagent

Dissolve 0.8 grams of basic fuchsin in 85 ml of distilled water. Add 1.9 grams of sodium metabisulfite and 15.0 ml of 1 N HCl. Place the solution in separatory funnel and shake at 2 hour intervals for a period of approximately 12 hours. Add 200 grams of a ctivated charcoal, shake for 1 minute and filter the clear solution. If the solution is still pink, add another 100 grams of charcoal and shake for an additional minute. Filter and store in a dark bottle. Solution should be clear (no pink coloration) for use.

Scott solution

Dissolve 2.0 grams of sodium bicarbonate and 20.0 grams of magnesium sulfate in water to a final volume of 1 liter. Add a pinch of thymol to retard the growth of molds.

SDS

Refer to Sodium lauryl sulfate.

1X SDS-Electrophoresis Running Buffer

Dilute 5X Tris-Glycine buffer to 1X and add 1.0 gram of SDS per liter of 1X Tris-Glycine. The pH should be 8.3 after dilution.

2X SDS Sample Buffer

Dissolve 1.52 grams of Tris base, 2.0 grams of SDS, 20 ml of glycerin, 2.0 ml of -mercaptoethanol and 1 mg of bromophenol blue to a final volume of 100 ml with water.

Siliconized pipettes

Pasteur pipettes can be siliconized by soaking them in a beaker containing 5% (v/v) dichlorodimethylsilane in chloroform for about 1 minute. Remove, drain and rinse several times with distilled water. Bake the pipettes at 180 ° C for 2 hours and cool before use.

Dichlorodimethylsilane and chloroform are both toxic and volatile. Use only in proper fume hood and keep all flames away from work area. Insure that all silicone and chloroform are removed from glassware before placing in an oven.

Silver nitrate solution (for electrophoresis staining)

Dissolve 0.15 grams of NaOH in 150 ml of water. Add 3.5 ml of concentrated NH_4OH and bring to a volume of 200 ml. Separately, dissolve 2.0 grams of silver nitrate in a final volume of 10 ml. With constant stirring, add 8.0 ml of the silver nitrate to the 200 ml of $NaOH/NH_4OH$.

This solution should be prepared immediately prior to use, and used within 30 minutes.

Dispose of this solution with copious flushing. It becomes explosive upon drying.

SM agar medium (Slime Mold medium of Sussman)

Dissolve 10.0 grams of glucose, 10.0 grams of peptone, 1.0 gram of yeast extract, 1.0 gram of $MgSO_4$, 1.5 grams of KH_2PO_4, 1.0 gram of $K2HPO_4$ and 20.0 grams of agar to a final volume of 1 liter. Heat to

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dissolve the agar, autoclave and dispense to petri plates.

Sodium acetate (MW 82.04)

1 M	Dissolve 82.04 grams of sodium acetate to a final volume of 1 liter with water or buffer.
0.02 M	Dissolve 1.64 grams of sodium acetate to a final volume of 1 liter with water or buffer.

Sodium acetate buffer

1 M pH 5.7	To 925 ml of 1 M sodium acetate, add 75 ml of 1 M acetic acid.

Sodium azide (MW 65.02)

0.01 M	Dissolve 0.065 grams of sodium azide to a final volume of 100 ml with water.
0.39% (w/v)	Dissolve 0.39 grams of sodium azide to a final volume of 100 ml with water or buffer.

Sodium barbitol

0.2% (w/v)	Dissolve 0.2 grams of sodium barbitol to a final volume of 100 ml with water.

Sodium bicarbonate ($NaHCO_3$ MW 84.0)

0.1 M	Dissolve 0.84 grams of $NaHCO_3$ to a final volume of 100 ml with water or buffer.

Sodium cacodylate buffer

0.2 M pH 7.4	Prepare a 0.2 M solution of cacodylic acid, sodium salt (MW 159.91). Dissolve 3.20 grams of cacodylic acid, sodium salt to a final volume of 100 ml with water. Adjust the pH to 7.4 with HCL.

Cacodylic acid contains arsenic. Handle properly.

Sodium carbonate (MW 106.0)

2% (w/v)	Dissolve 2.0 grams of sodium carbonate to a final volume of 100 ml with 0.1 N NaOH.
0.1 N NaOH	Used for Lowry Protein assay.

Sodium chloride (MW 58.44)

M	For a molar solution of sodium chloride, dissolve 58.44 grams of NaCl to a final volume of 1 liter with water or buffer. For corresponding dilutions, multiply the weight by the molarity required. For example, for 0.05 M, multiply 58.44 by 0.05 or 2.92 grams/liter.
%	For % solutions, they are invariably w/v. For a 1 % (w/v) solution, dissolve 1.0 gram of NaCl to a final volume of 100 ml with water or buffer. Multiply the weight by a corresponding change in % for other concentrations.
200,300,400 mOsM	Osmoles for NaCl are calculated as twice the molar concentration. Thus, a 200 mOsM solution would be .100 M NaCl. Likewise, 300 mOsM would be .150 M and 400 mOsM would be .200 M NaCl.

Sodium citrate (MW 294.10)

0.09 M	Dissolve 2.65 grams of sodium citrate to a final concentration of 100 ml with water.

Sodium citrate/formaldehyde (for silver stained proteins)

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Dissolve 5.0 grams of sodium citrate in 800 ml of water, add 5.0 ml of concentrated formalin (37% formaldehyde solution) and dilute to 1 liter with water.

Sodium deoxycholate (Deoxycholic acid, sodium salt MW 392.58)

0.15% (w/v)	Dissolve 150 mg of deoxycholic acid, sodium salt to a final volume of 100 ml with water.

Sodium dithionite ($Na_2S_2O_6 \cdot 2H_2O$ MW 242.16)

.1 mg/ml	Dissolve 10 mg of sodium dithionite in 100 ml of water just prior to use. Alternatively, to reduce a solution, the dry powder can be added as needed. Sodium dithionite should be stored at -20 ° C .

Sodium fluoride (NaF MW 42.0)

0.1 M	Dissolve 4.2 grams of NaF to a final volume of 1 liter with water.

Sodium lauryl sulfate (SDS or SLS MW 288.38)

0.1% (w/v)	Dissolve 0.1 grams of SDS to a final volume of 100 ml with water or buffer. Mix by gentle stirring, do not shake.
10% (w/v)	Dissolve 10 grams of SDS to a final volume of 100 ml with water.

SDS should not be inhaled in its powder form. When weighing, use a mask, or better, a hood.

Sodium malonate (MW 104.0)

0.6 M	Dissolve 2.49 grams of malonic acid, sodium salt, to a final volume of 25 ml with water or buffer.

Sodium perchlorate ($NaClO_4 \cdot H_2O$ MW 140.47)

1 M	Dissolve 14.01 grams of sodium perchlorate to a final volume of 100 ml with water or buffer.

Sodium phosphate, monobasic ($NaH_2PO_4 \ H_2O$ MW 137.99)

1 M	Dissolve 14.01 grams of sodium perchlorate to a final volume of 100 ml with water or buffer.
0.01 M	Dissolve 1.38 grams of monobasic sodium phosphate to a final volume of 1 liter.

Sodium phosphate, dibasic ($Na_2HPO_4 \cdot 7H_2O$ MW 268.07)

1 M	Dissolve 268.07 grams of dibasic sodium phosphate to a final volume of 1 liter.
0.2 M	Dissolve 53.61 grams of dibasic sodium phosphate to a final volume of 1 liter.
0.01 M	Dissolve 2.68 grams of dibasic sodium phosphate to a final volume of 1 liter.

Sodium phosphate buffer

These are the most common buffers used in biology. They are produced by adding equimolar solutions of KH_2PO_4 and Na_2HPO_4. Equal volumes of the two will yield a pH of 7.0, while sodium phosphate will increase the pH. Increased volumes of potassium phosphate will decrease th e pH. The pH can be adjusted from 5.4 to 8.2.

If a pH of 7.0-8.2 is desired, start with 500 ml of sodium phosphate and add potassium phosphate while stirring and monitoring the pH with a pH meter until the desired pH is reached.

If a pH of 5.4-7.0 is desired, start with 500 ml of potassium phosphate and add sodium phosphate until

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the desired pH is reached.

Typically, the molarity of the buffer will range from 0.01 to 0.1 M. Use the appropriate molarity of KH_2PO_4 and Na_2HPO_4. That is, if 0.05 M buffer is desired, use 0.5 M KH_2PO_4 and 0.5 M Na_2HPO_4 as directed above.

Sodium potassium phosphate buffer

Refer to Sodium phosphate buffer.

Sodium pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$ MW 446.06)

10 mM	Dissolve 0.446 grams of $Na_4P_2O_7 \cdot 10H_2O$ to a final volume of 100 ml with water.

Sodium succinate (MW 270.16)

0.6 M	Dissolve 16.2 grams of succinic acid, sodium salt to a final volume of 100 ml with water or buffer.

Sorbitol (MW 182.17)

0.33 M	Dissolve 60.12 grams of sorbitol to a final volume of 1 liter with water or buffer.

Sorenson phosphate buffer

Refer to sodium phosphate buffer.

0.2 M pH 7.5	Dissolve 24.14 grams of Na_2HPO_4 and 4.08 grams of KH_2PO_4 in 800 ml of water. Dilute to a final volume of 1 liter.

Subbing solution (slides)

Refer to chrom alum gelatin.

Sucrose (MW 342.3)

1.0 M	Dissolve 34.2 grams of sucrose to a final volume of 100 ml with water or buffer. For other molarities, multiply the weight by the required molar concentration. For example, for 0.25 M sucrose, weight 34.2 x 0.25 or 8.55 grams to a final volume of 100 ml.
40% (w/v)	Dissolve 40 grams of sucrose to a final volume of 100 ml with water or buffer. Dilute this solution for lower percent requirements. If using for sucrose density gradients, the sucrose should have 0.1 ml of diethylpyrocarbonate added, the solution brou ght to a boil for 3-5 minutes and cooled before use. This will eliminate RNAase, which would otherwise be a contaminant of the solution. Store all sucrose solutions in a refrigerator

Sulfuric Acid (H2SO4 MW 98.08)

Caution: Sulfuric acid is extremely caustic and will cause severe burns. It must always be added to the water, when making dilutions. Upon addition to water or alcohol, heat will be generated while the solution will contract in volume. Use extreme care in handling this acid.

Concentrated H2SO4 is 17.8 M or 35.6 N. 1.09 N Add 30.6 ml of concentrated sulfuric acid slowly, with constant stirring, and with adequate protection from splashes, to approximately 800 ml of water. Cool and make up volume to 1 liter with water.

Sulfurous acid (for Feulgen Reaction)

Add 1.0 ml of concentrated HCl and 0.4 grams of sodium bisulfite to 100 ml of distilled water. This solution should be made fresh prior to use. It does not store well.

Swabbing detergent

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For tissue culture purposes, use a non-toxic detergent designed for surgical scrubbing. e.g. Phisohex, Betadine or equivalent. For most routine swabbing, 70% (v/v) ethanol is sufficient and has the advantage that it will leave no residue.

TEMED (N,N,N',N'-tetramethylethylenediamine)

Catalyst for PAGE. Use directly and add 10 l TEMED per 15 ml of gel solution.

Toluidine blue

0.1% (w/v)	Dissolve 0.1 grams of toluidine blue in 10 ml of ethanol and add water or citrate buffer (pH 6.8-7.2) to a final volume of 100 ml.

Trichloroacetic acid (TCA CCl3COOH MW 163.4)

Extremely caustic acid. Handle with care.

72% (w/v)	Dissolve 72 grams of TCA to a final volume of 100 ml. TCA is hydroscopic and will readily absorb water. The solid crystals will become liquid if the stock bottle is placed in warm water, with a loose cap (melting point 57-58 ° C. It is easier to handle as a liquid. Storage of solutions greater than 30% (w/v) are not recommended as decomposition is rapid. Therefore these solutions should be made as needed.

Tris buffer

	There are many variations on the basic Tris-HCl buffer combination, most of which are commercially available. Solutions with EDTA are known as TE buffers, while solutions with EDTA and acetic acid are known as TAE buffers. The terminology varies with the author, with Tris buffer being used to mean Tris-HCl solutions. Sigma Chemical Co., St. Louis, carries a full line of the buffers marketed under the tradename of Trizma (base and HCl). The basic buffer is a combination of Tris (tris(hydroxymethyl)aminomethane) and HCl acid. These are sometimes referred to as Tris-base and Tris-HCl solutions. Tris buffers should not be used below a pH of 7.2 or above a pH of 9.0. Tris buffers are also ex tremely temperature senstive. Directions are given for room temperature (25 ° C). The pH will decrease approximately 0.028 units for each degree decrease in temperature.
1 M	Dissolve 121 grams of Tris in 800 ml of distilled water. Adjust the pH with concentrated HCl. Dilute to a final volume of 1 liter. Lower required molarities can be diluted from this stock or mixed as combinations of lower molarities of Tris and HCl. It is important to measure the pH at the temperature an d molarity that will be used in the final analysis.

Tri-Glycine buffer

5X	Dissolve 15.1 grams of tris base and 72.0 grams of glycine to a final volume of 1 liter. For use, dilute 1 part 5X buffer with 4 parts water.

Trypan blue

0.2 % (w/v)	Dissolve 0.2 grams of trypan blue to a final volume of 100 ml with water.

Trypsin

0.25%	0.25% Dissolve 0.25 grams of crude trypsin in PBSA to a final volume of 100 ml. Cold sterilize by filtration. Alternatively, purchase pre-diluted crude trypsin, sold as 1:250 which is pre-sterilized as well.

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Note:	When using trypsin for tissue disaggregation, it must be subsequently inhibited by the use of serum in the culture media, or by the addition of soya bean trypsin inhibitor.

Trypticase soy broth

Add 17.0 grams of trypticase peptone, 3.0 grams of phytone peptone, 5.0 grams of sodium chloride, 2.5 grams of dipotassium phosphate and 2.5 grams of glucose to 1 liter of water. Adjust the pH to 7.3, and autoclave.

Tween 20 or 80 (Polyoxyethylene sorbitan mono-oleate)

1% (v/v)	Add 1.0 ml of Tween to 90 ml of water. Mix and dilute to a final volume of 100 ml with water. Note that Tween is extremely viscous and care must be taken to accurately pipette 1.0 ml. Wipe the outside of the pipette before dispensing.

Uranyl acetate (MW 424.19)

5% (w/v)	Dissolve 5.0 grams of uranyl acetate to a final volume of 100 ml in 50% (v/v) ethanol. Store in the dark at room temperature. Allow at least 24 hours for the uranyl acetate to completely dissolve. This solution will keep for about 3 months.

Urea (MW 60.06)

2.5 M	Dissolve 15.02 grams of urea to a final volume of 100 ml with water or buffer.
10 M	Dissolve 60.06 grams of urea to a final volume of 100 ml with water or buffer.
14 M	Dissolve 84.08 grams of urea to a final volume of 100 ml with water or buffer.

Viability stain

Refer to Trypan blue