EDO UNIVERSITY IYAMHO

Department of Biochemistry

I 313 Techniques in Biochemical Research

Department of Biochemistry

Instructor I: *Dr. U. Usunobun* email: usunobun.usunomena@edouniversity.edu.ng

Instructor II: *Dr. S. K. Rahamon*email: sheu.rahamon@edouniversity.edu.ng

Lectures: Monday, 9 am ï 11 am, LT6, Phone: 08034174871, 08067420607

Office hours: Wednesday, 10 am to 12 pm, Office: 1st floor, New College Building

General overview of lecture: This aspect of Biochemistry is intended to introduce Biochemistry students to basic techniques in Biochemistry.

Prerequisites: No prerequisite biochemistry course is required.

Learning outcomes: At the end of the lectures, students should be able to:

1. List commonly used equipment in Biochemistry laboratory
2. Explain the principle(s) of commonly used equipment in Biochemistry laboratory
3. Select (and justify the choice) appropriate equipment for various biochemical analysis
4. Assess the performance of at least, one equipment in Biochemistry laboratory

Assignments: Students will be given at least 2 individual homework and 1 oral presentation throughout the course in addition to a Mid-Term Test and a Final Examination. Home works are due at the beginning of the class on the due date. Home works are organized and structured as preparation for the midterm and final examination, and are meant to be a studying material for both examinations.

Grading: We will assign 10% of this class grade to homework, 10% to oral presentation and 10% for the mid-term test. The final examination, which will be comprehensive, shall be 70%.

Textbooks: The recommended textbooks for the class are as stated:

Title: *Tietz Textbook of Clinical Chemistry and Molecular Diagnostics*

Author: Burtis& others

Publisher: Elsevier Saunders, Missouri, USA

ISBN-13: 978-007216-0189-2

ISBN-10: 0-7216-0189-8

Year: 2006

Title: Understanding Bio-analytical Chemistry; Principles and Applications

Author: V. A. Gault and N. H. McClenaghan

Publisher: John Wiley & Sons Ltd, UK

ISBN: 978-0-470-02906-0 (HB)

978-0-470-02907-7 (PB)

CENTRIFUGATION

A centrifuge of some kind is found in every biochemistry laboratory. Centrifuges have many applications, but they are used primarily for the preparation of biological samples and for the analysis of the physical properties of biomolecules, organelles, and cells.

Centrifugation is carried out by spinning a biological sample at a high rate of speed, thus subjecting it to an intense force (artificial gravitational field). Most centrifuge techniques fit into one of two categoriesð **preparative centrifugation** or **analytical centrifugation.**

A preparative procedure is one that can be applied to the separation or purification of biological samples (cells, organelles, macromolecules, etc.) by sedimentation. Analytical procedures are used to measure physical characteristics of biological samples. For example, the purity, size, shape, and density of macromolecules may be defined by centrifugation.

Centrifugation is used to separate all types of particle based upon their sedimentation properties. The sedimentation properties of particles depend on a number of different factors including size, density and shape. However, both density and shape vary significantly depending on the composition of the solution in which the particles are suspended. Particles are separated primarily on the basis of either their density (isopycnic separations) or size (differential pelleting and rate-zonal separations)

BASIC PRINCIPLES OF CENTRIFUGATION

A particleð whether it is a floating solid, a precipitate, a macromolecule, or a cell organelleð is subjected to a centrifugal force when it is rotated at a high rate of speed. The **centrifugal force,** *F*, is defined by Equation

$F = m\omega^2 r$

Where:

F = intensity of the centrifugal force

m = effective mass of the sedimenting particle

ω = angular velocity of rotation in rad/sec

r = distance of the migrating particles from the central axis of rotation

The force on a sedimenting particle increases with the velocity of the rotation and the distance of the particle from the axis of rotation.

INSTRUMENTATION FOR CENTRIFUGATION

The basic centrifuge consists of two components, an electric motor with drive shaft to spin the sample and a **rotor** to hold tubes or other containers of the sample.

A wide variety of centrifuges is available, ranging from a low-speed centrifuge used for routine pelleting of relatively heavy particles to sophisticated instruments that include accessories for making analytical measurements during centrifugation. Here we will describe three types, the low-speed or clinical centrifuge; the high speed centrifuge, including the ñmicrofugeò; and the ultracentrifuge.

Low-Speed Centrifuges

Most laboratories have a standard low-speed centrifuge used for routine sedimentation of relatively heavy particles. The common centrifuge has a maximum speed in the range of 4000 to 5000 rpm.

These instruments usually operate at room temperature with no means of temperature control of the samples. Two types of rotors, **fixed angle** and **swinging bucket,** may be used in the instrument. Centrifuge tubes or bottles that contain 12 or 50 mL of sample are commonly used.

Low-speed centrifuges are especially useful for the rapid sedimentation of coarse precipitates or red blood cells. The sample is centrifuged until the particles are tightly packed into a **pellet** at the bottom of the tube. The upper liquid portion, the **supernatant,** is then separated by decantation.

High-Speed Centrifuges

For more sophisticated biochemical applications, higher speeds and temperature control of the rotor chamber are essential. The operator of this instrument can carefully control speed and temperature, which is especially important for carrying out reproducible centrifugations of temperature-sensitive biological samples. Rotor chambers in most instruments are maintained at or near 4°C.

Three types of rotors are available for high-speed centrifugation: the fixed angle, the swinging-bucket, and the vertical rotor (Figure Aï C). Fixed-angle rotors are especially useful for differential pelleting of particles (Figure A). In swinging-bucket rotors (Figure B), the sample tubes move to a position perpendicular to the axis of rotation during centrifugation. These are used most often for density gradient centrifugation. In the vertical rotor (Figure C), the sample tubes remain in an upright position. These rotors are used often for gradient centrifugation.

Prior to the early 1990s, rotors were constructed from metals such as aluminum and titanium. Although metal rotors have great strength, they do have several disadvantages: they are very heavy to handle, they are not corrosion-resistant, and they become fatigued with use.

Rotors are now available that are fabricated from carbon-fiber composite materials. They have several advantages over heavy metal rotors. These new rotors are 60% lighter than comparable aluminum and titanium rotors. Because of the lighter weight, acceleration and deceleration times are reduced; thus, centrifuge run times are shorter. This also results in lower service and maintenance costs. Instruments are equipped with a brake to slow the rotor rapidly after centrifugation.

Widely used in the category of medium-speed centrifuges is the **microfuge**. These instruments, which are designed for the bench top, are used for rapid pelleting of small samples. Fixed-angle rotors are able tohold up to eighteen 1.5- or 0.5-mL tubes. The maximum speed of most commercialmicrofuges is between 12,000 and 15,000 rpm. Some instruments can accelerate to full speed in 6 secondsand decelerate within 18 seconds. Most instruments have a variable speed controland a momentary pulse button for minispins.

The preparation of biological samples almost always requires the use of a high-speed centrifuge. High-speed centrifuges may be used to sediment: (1) cell debris after cell homogenization, (2)

ammonium sulfate precipitates of proteins, (3) microorganisms, and (4) cellular organelles such as chloroplasts, mitochondria, and nuclei.

Ultracentrifuges

The most sophisticated of the centrifuges are the **ultracentrifuges.** Because of the high speeds attainable, intense heat is generated in the rotor. The spin chamber must thus be refrigerated and placed under a high vacuum to reduce friction. The sample in a cell or tube is placed in a rotor, which is then driven by an electric motor. Although it is rare, metal rotors sometimes break into fragments when placed under high stress. The rotor chamber on all ultracentrifuges is covered with a protective steel armor plate. The drive shaft of the ultracentrifuge is constructed of a flexible material to accommodate any ñwobbleò of the rotor due to imbalance of the samples. It is still important to counterbalance samples as carefully as possible before inserting them in the rotor.

The previously discussed centrifugesð the low, medium, and high speedð are of value only for preparative work, that is, for the isolation and separation of precipitates and biological samples. Ultracentrifuges can be used both for preparative work and for analytical measurements. Thus, two types of ultracentrifuges are available: **preparative models,** primarily used for separation and purification of samples for further analysis, and **analytical models,** which are designed for performing physical measurements on the sample during sedimentation.

Two of the most versatile models are Beckman Optima MAX and TLX microprocessor-controlled tabletop ultracentrifuges. With a typical fixed-angle rotor, which holds six 0.2- to 2.2-mL samples, the instruments can generate 150,000 rpm.

Analytical ultracentrifuges have the same basic design as preparative models, except that they are equipped with optical systems to monitor directly the sedimentation of the sample during centrifugation. The first commercial instrument of this type was the Beckman Model E, introduced in 1947.

Beckman Optima TLX ultracentrifuge.

For analysis, a sample of nucleic acid or protein (0.1 to 1.0 mL) is sealed in a special analytical cell and rotated. Light is directed through the sample parallel to the axis of rotation, and measurements of absorbance by sample molecules are made. (The Beckman instrument can scan the sample over the wavelength range 190 to 800 nm.) If sample molecules have no significant absorption bands in the wavelength range, then optical systems that measure changes in the refractive index may be used. Optical systems aided by computers are capable of relating absorbance changes or index of refraction changes to the rate of movement of particles in the sample. The optical system actually detects and measures the front edge or moving boundary of the sedimenting molecules. These measurements can lead to an analysis of concentration distributions within the centrifuge cell.

APPLICATIONS OF CENTRIFUGATION

Preparative Techniques

Centrifuges in undergraduate biochemistry laboratories are used most often for preparative-scale separation procedures. This technique is quite straightforward, consisting of placing the sample in a tube or similar container, inserting the tube in the rotor, and spinning the sample for a fixed period. **(Filled centrifuge tubes or bottles must be weighed and balanced before centrifugation.**

Tubes across from each other in the rotor should have approximately the same weight.) The sample is removed and the two phases, pellet and supernatant(which should be readily apparent in the tube), may be separated by careful decantation.Further characterization or analysis is usually carried out on the individual phases. This technique, called **velocity sedimentation centrifugation,** separates particles ranging in size from coarse precipitates to cellular organelles.

Relatively heavy precipitates are sedimented in low-speed centrifuges, whereas lighter organelles such as ribosomes require the high centrifugal forces of an ultracentrifuge.

Much of our current understanding of cell structure and function depends on separation of subcellular components by centrifugation. The specific method of separation, called **fractional centrifugation,** consists of successive centrifugations at increasing rotor speeds. Figure below illustrates the fractional centrifugation of a cell homogenate, leading to the separation and isolation of the common cell organelles. For most biochemical applications, the rotor chamber must be kept at low temperatures to maintain the native structure and function of each cellular organelle and its component biomolecules. A high-speed centrifuge equipped with a fixed-angle rotor is most appropriate for the first two centrifugations at 600 x g and 20,000 x g. After each centrifuge run, the supernatant is poured into another centrifuge tube, which is then rotated at the next higher speed. The final centrifugation at 100,000 x g to sediment microsomes and ribosomes must be done in an ultracentrifuge. The 100,000 x g supernatant, the **cytosol,** is the soluble portion of the cell and consists of soluble proteins and smaller molecules.

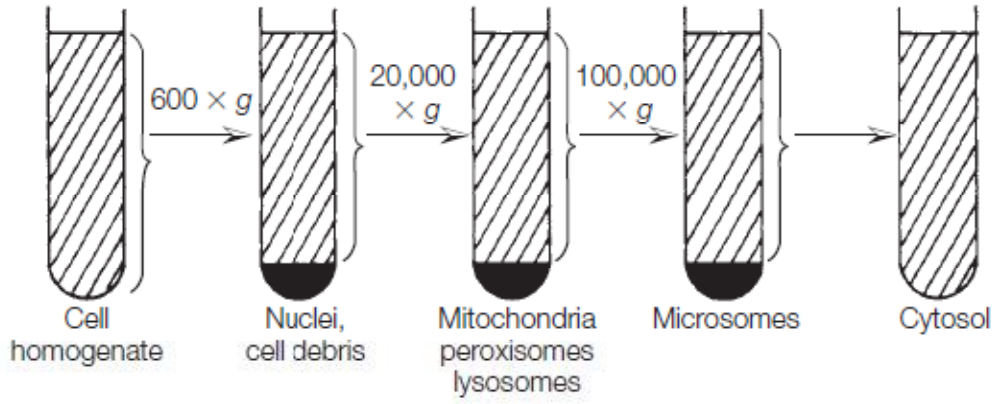


FIGURE 4.11 Fractional centrifugation of a cell homogenate. See text for details.

CARE OF CENTRIFUGES AND ROTORS

Centrifuge equipment represents a sizable investment for a laboratory, so proper maintenance is essential. In addition, poorly maintained equipment is especially dangerous. Since many kinds of instruments are now available, specific instructions will not be given here, but general guidelines are outlined below.

1. Carefully read the operating manual or receive proper instructions before you use any centrifuge.

2. Check the rotor chamber for cleanliness and for damage. Clean with soap and warm water, rinse with distilled water, and dry.

3. Select the proper operating conditions on the instrument. If refrigeration is necessary, set the temperature to the appropriate level and allow 1 to 2 hours for temperature equilibration.

4. Select the proper rotor. Many sizes and types are available. Follow guidelines or consult your instructor.

5. Be sure the rotor is clean and undamaged. Observe any nicks, scratches, or other damage that may cause imbalance. If dirty, the rotor should be cleaned with warm water and a mild, nonbasic detergent. A soft brush can be used inside the cavities. Rinse well with distilled water and dry. Scratches should not be made on the surface coating, as corrosion may result.

6. Filled centrifuge tubes or bottles should be weighed carefully and balanced before centrifugation.

7. Rotor manufacturers provide a maximum allowable speed limit for each rotor. Do not exceed that limit.

8. Keep an accurate record of centrifuge and rotor use. Just as your automobile needs service after a certain number of miles, the centrifuge should be serviced after certain intervals of use. Centrifuge maintenance is usually determined by hours of use and total revolutions of the rotor. It is also essential to maintain a record of the use for each rotor. Metal rotors weaken with use, and the maximum allowable speed limit decreases. Rotor manufacturers usually provide guidelines for decreasing the allowable speed for a rotor (derating a rotor).

9. If an unusual noise or vibration develops during centrifugation, immediately turn the centrifuge off.

10. Carefully clean the rotor chamber and rotor after each centrifugation.

A, Bench-top Eppendorf centrifuge with samples arranged symmetrically around the axis of the rotor. B, Semi-preparative, refrigerated centrifuge with a rotor for Eppendorf tubes. C, Preparative, refrigerated centrifuge with a rotor for the handling of volumes up to 6 litres. D, Semi-preparative rotor with lid. E, Preparative rotor with lid.

Centrifuge tubes.

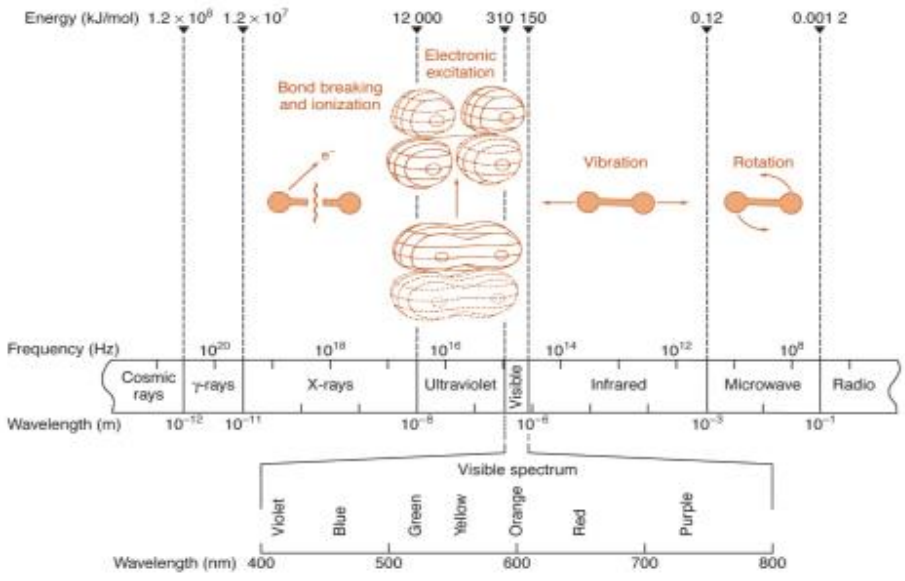
A, Micro-ultracentrifuge. B, Rotors compatible with the micro-ultracentrifuge. C, Semi-preparative ultracentrifuge. D, Rotors compatible with the semi-preparative ultracentrifuge.

PHOTOMETRY

Photometry is the measurement of light (radiant energy). In the laboratory, many biochemical determinations are based on measurements of light that is emitted, transmitted, absorbed, scattered or reflected. The measurement of the radiant energy is usually under controlled conditions.

Biochemical determinations involving photometry is carried out at specific wavelength (distance between two peaks of light), which is expressed in nanometers (nm), millimicrons (mε) or angstrom (Å). These units are inter-convertible; 1 nm = 1 mε = 10 Å = 10^{-9} m

Spectra of light required in photometry are ultraviolet (UV), visible and infrared (IR).



Regions of Electromagnetic Spectrum

Photons

Light is composed of discrete energy known as photons. These photons are related to the frequency by the equation:

$E = hv$ equation 1

Where:

E = energy in ergs

v = frequency of light in cycles per second

h = Planckôs constant (6.62×10^{27} erg second)

On the other hand, the frequency of light is related to the wavelength (ę) by the equation:

$v = c/ę$ equation 2

Where:

v = frequency of light in cycles per second

c = speed of light in a vacuum (3×10^{10} cm/s)

ę = wavelength in centimeters

Substituting for v in equation 1, the equation becomes:

$E = hc/ę$ equation 3

Therefore, energy of light is 1/Ŭto the wavelengthi.e. radiation energy at 200 nm > 750 nm

Applications of photometry

Photometry is the most common analytical technique in biochemistry laboratory. Its principles are applied in:

- Spectrophotometry
- Colorimetry
- Atomic absorption spectrophotometry
- Turbidimetry
- Flame emission spectrophotometry and,
- Reflectance photometry among others

SPECTROPHOTOMETRY