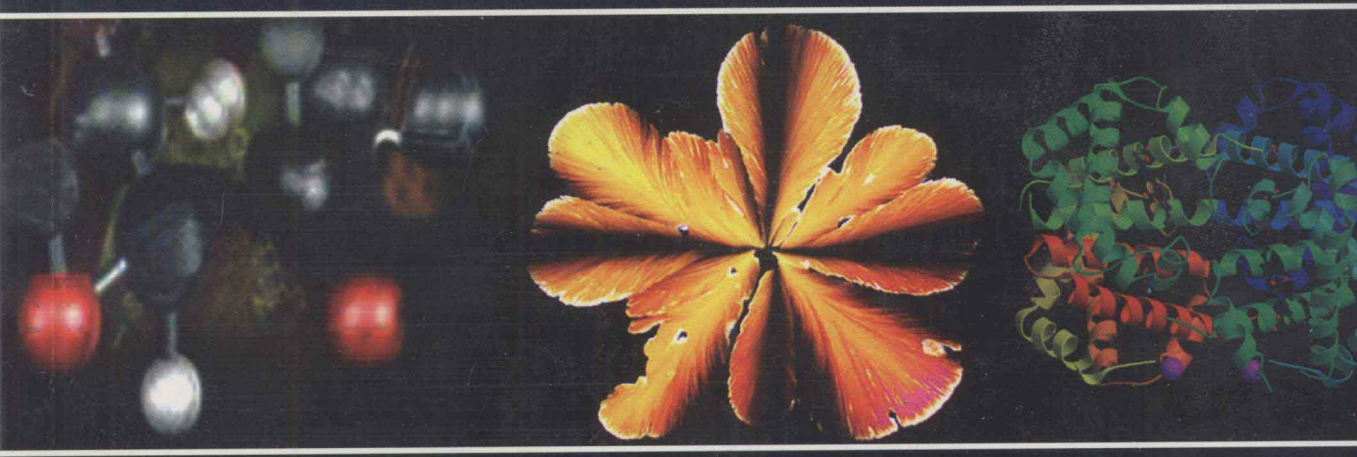

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FIRST EDITION

Chief Editors
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HE Chunyan , GUAN Yaqun, ZHANG Baifang, GE Yinlin

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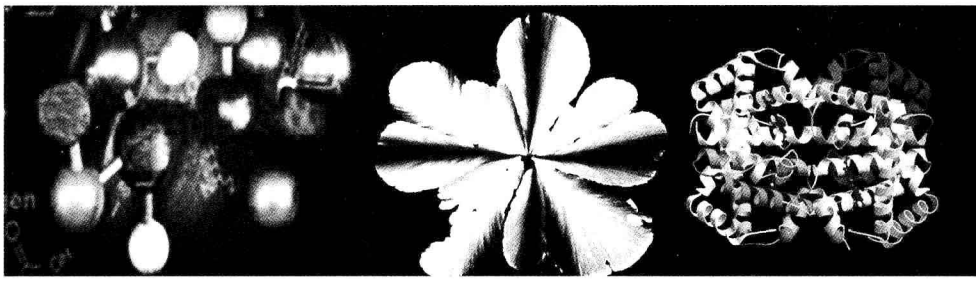

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PREFACE

Progress in life science, including medical science, is mainly made by observation and experiment, especially biochemistry and molecular biology experiment. So, learning the basic biochemical techniques and methods is essential to medical students and the students of other related disciplines.

The first edition of this manual presents shortened versions of the basic biochemistry methods designed for use at the lab bench in a biochemistry and molecular biology laboratory. It is primarily intended for undergraduate medical students, and hopefully it will be a useful reference book for graduate students and teaching staff. The objectives for the course are for students to learn modern biochemical laboratory methodologies and techniques, develop the ability to perform experiments successfully and independently, properly interpret the data and write scientific laboratory reports. Students who learn biochemistry as a part of medical science should be able to understand the concepts and methods of not only the conventional clinical laboratory tests but also the recently developed tests. For this purpose, we have made this manual with incorporation of some clinical laboratory test kits. All of the experiments have been performed many times in our own laboratory. We hope that they will provide a reliable training of the most important and commonly used techniques of contemporary biochemistry.

We express our appreciation to our colleagues in the Department of Biochemistry and Molecular Biology, Wuhan University School of Medicine, who have contributed ideas, techniques and experiences to the manual. We are really grateful to all the experts, especially those from other universities, who made big contribution to the compiling of various chapters.

This manual is not a complete version. It should be continually modified and updated. We would be very grateful if the users of this manual could bring good feedbacks for the development of this manual.

Yu hong and Huang xinxiang

July, 2008

Requirements

Lab safety

1. Eating, drinking, smoking or chewing gum in the laboratory is strictly prohibited.
2. Always wear lab-coat, sandals are not allowed in the laboratory.
3. Keep your work area clean. When chemicals are spilled they should be wiped as soon as possible. Be aware of objects that can burn or give electrical shocks.
4. Mouth pipetting is not allowed.
5. Do not turn on an instrument until you have read instructions and consulted the instructors for the use of equipment. If any equipment malfunction is noted, report this immediately.
6. Anyone carrying out these protocols will encounter the following hazardous materials: (1) toxic chemicals and carcinogenic or teratogenic reagents, (2) pathogens and infectious biological agents. We emphasize that users must proceed with the prudence and precaution associated with good laboratory practice. Use chemicals with high vapor pressure only in the hood. Handle and dispose of hazardous chemicals properly. Disposal containers are provided.
7. In case of an accident, notify an instructor immediately. For any chemicals splashed in the eye, hold the eye open and flush immediately with cold water using the eye wash. For chemicals spilled on the skin or splashed into the mouth, flush with large amounts of cold water. For burns, flush with cold water and contact an instructor.

Attendance policy and lab reports

Students are expected to attend every lab, and to arrive promptly and well prepared. A student who is absent without the prior permission of the instructor or does not have documented excuse will receive 0 points on the lab report for that experiment.

All lab reports must be done on an individual basis. You will be given instructions about the format and the information needed for each experiment. Laboratory reports must include the following: experiment title, date (s) experiment performed, experimental results (data, method of calculations, tables, graphs, figures, significance of the data), discussions.

Content

Chapter I

Spectrophotometry

Spectrometry is a qualitative and quantitative technology based on emission spectra, absorption spectra or scattering spectra of the material. Spectrometry can be classified in many different types depended on the form of spectra, including atomic emission spectrometry, flame photometry and fluorescence spectrometry based on the character of emission spectra, UV and visible spectrophotometry based on the absorption spectra, atomic absorption and infrared spectroscopy based on scattering spectral character.

Spectrophotometry is based on the reflection or transmission properties of a substance. It has some advantages, such as simple, rapid, high sensitivity, accuracy, and selectivity. So, it is one of the most widely used analytical techniques in biochemistry laboratory and clinical research.

1.1 Basic Concepts and Principle of Spectrophotometry

Light is a kind of electromagnetic wave. The light can be seen by our naked eyes is really a very small portion of the electromagnetic spectrum (Figure 1-1) within the wavelength range between approximately 380 nm and 760 nm. The visible light could be split into many colors by a prism. Each color is signed by the wavelength of light. Any solution that contains a subtance that absorbs the visible light will appear a color. Light with wavelengths longer than 760 nm or shorter than 380 nm is invisible. Ultraviolet is a region of the electromagnetic spectrum that has a wavelength range from 375 nm to 12.5 nm. Ultraviolet-Visible spectrophotometry using lights in the visible range and adjacent near ultraviolet (UV) range (200 ~ 375 nm) is discussed in this chapter.

1.1.1 Absorption spectrum

Each substance has its own characteristic spectrum. The light absorption of the same substance at different wavelengths is different, and light absorption of different substances at the same wavelength is also different. Spectrophotometry is based on this selective absorption of

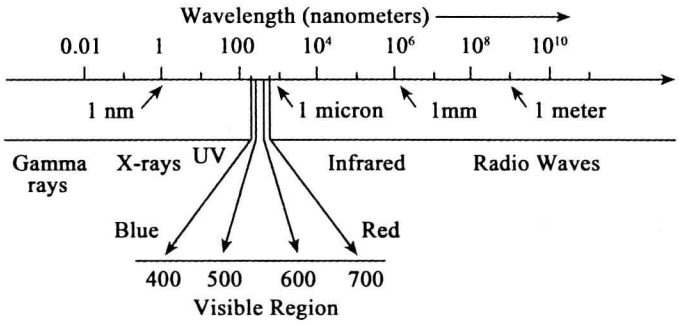

Fig. 1-1 The electromagnetic spectrum

light by a substance. Because the extent to which a sample absorbs light depends upon the wavelength of light, spectrophotometry is performed using monochromatic light.

To clearly describe the selective absorption of light by a substance, an absorption spectrum is usually plotted, which shows how the absorption of light varies with the wavelength of the light (figure 1-2). The extent of light absorption is commonly referred to as absorbance (A) or extinction (E). The absorption spectrum is a plot of absorbance vs wavelength and is characterized by the wavelength at which the absorbance is the greatest (λ_{max}). The λ_{max} is the characteristic of each substance and provides information on the electronic structure of an analyte. Unknown substance can be identified by their characteristic absorption spectrum and λ_{max} (qualitative analysis).

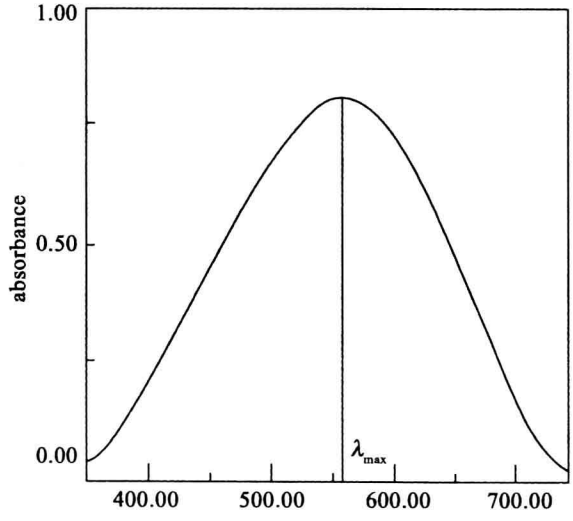

Fig. 1-2 Absorption spectrum

1.1.2 Basic laws of absorption of light—Lambert-Beer's Law

The quantitative analysis of spectrophotometry is based on the basic laws of absorption of light—Lambert-Beer's law. When monochromatic light (light of a specific wavelength) passes through a solution there is usually a quantitative relationship between the solute concentration and the absorption of the light, which is described by the Lambert-Beer's law. Therefore the concentration of solute can be measured by determining the extent of absorption of light at the appropriate wavelength. In order to obtain the highest sensitivity and to minimize deviations in quantitative measurement, spectrophotometric measurements are usually made using light with a wavelength of λ_{max}.

For a uniform absorbing medium the portion of the light passing through it called the transmittance, T. $T = I/I_0$, where I_0 is the intensity of the incident light, I is the intensity of transmitted light. The absorbance of the light is equal to the logarithm of the reciprocal of the transmittance: $A = \lg (1/T)$.

According to Lambert-Beer's law, when a ray of monochromatic light passes through an absorbing solution, absorbance of the solution is directly proportional to the concentration of the absorbing substance and the depth of the solution through which the light passes. Equation for Lambert-Beer's law is: $A = \lg 1/T = KCL$, where K is absorption coefficient (the proportionality constant that depends on the absorbing substance, wavelength of light and the temperature), C is the concentration of the substance absorbing light, L is the length of the path of the light. A is dimensionless. When length 'L' is in centimeter and concentration 'C' = 1mol/L, the absorbance is equal to 'ε' (molar extinction coefficient), which is written as $\varepsilon^{1mol/L}$ and has a dimension of 1 $mol/L^{-1}cm^{-1}$. Since the molar extinction coefficient may be very large, an alternative is $E^{1\%}$, which represents the extinction given by 1cm thick sample of a 1% solution of the substance. If the $\varepsilon^{1mol/L}$ or $E^{1\%}$ is known, the amount of the substance can be quantified, $C = A/\varepsilon$, or $C = A/E^{1\%}$.

If the Lambert-Beer's law is obeyed, a plot of absorbance against concentration gives a straight line passing through the origin. Sometimes, a non-linear plot is obtained of absorbance against concentration. This is probably not satisfied with one or more of the following prerequisites of Lambert-Beer's law.

1. Light must be of a narrow wavelength range, preferably monochromatic.
2. The wavelength of light used should be λ_{max}.
3. The solution should be stable and uniform. There must not be ionization, association, dissociation of the solute during the process of spectrophotometric measurement.
4. Solution concentration is not too high ($A = 0.15 \sim 0.7$).

1.2 Measurement of Light Absorption

1.2.1 Components of spectrophotometer

A spectrophotometer is employed to measure the light absorption of a sample. There are many kinds of spectrophotometers. All the spectrophotometers employ the basic components illustrated in Figure 1-3.

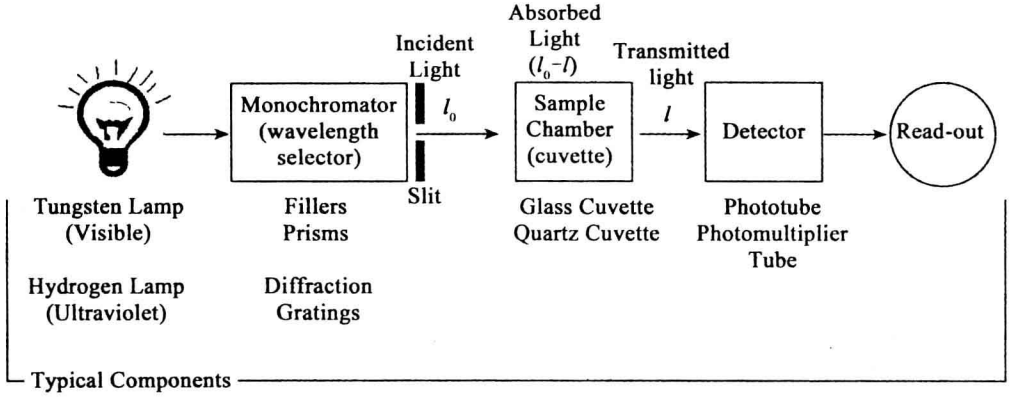

Fig. 1-3 The main components of a spectrophotometer

1.2.1.1 Light sources

The source of visible light is tungsten lamp that emits light in the range of 340 to 900 nm. Some spectrophotometers employ an additional deuterium lamp emitting light in the range of 200 to 360 nm for spectral analysis in the UV range.

1.2.1.2 Wavelength selector

(1) Monochromator A monochromator is an optical device that transmits a mechanically selectable narrow band of wavelengths of light. Selection of wavelengths may be done by the use of optical filters, prisms of quartz, or diffraction grating. However, it is not feasible to have a light of a single wavelength. The monochromatic light produced by monochromator is a light that has its maximum emission at a specific wavelength, with progressively less energy at longer and shorter wavelengths. Therefore, the purer the monochromatic light is, the sensitive the measurement is.

(2) Slit system The spectrophotometer places narrow slit in the light path that confine the light beam to a narrow path and also help to exclude light from extraneous sources. Then the intensity of the incident light can be adjusted.

1.2.1.3 Sample container-cuvettes

Glass cuvette is for visible light measurement. Quartz cuvette is for UV measurement (below 340 nm). Absorbance characteristics of cuvettes should always be checked in order to obtain a standardized set for spectrophotometric measurement.

1.2.1.4 Detector systems

Vacuum phototubes and photomultiplier tube are usually used to detect the transmitted light. Most detectors have a scale that reads both in absorbance units, which is a logarithmic scale, and in % transmittance, which is an arithmetic scale. The absorbance scale is normally read directly for calculation of sample concentration.

1.2.2 Using a spectrophotometer

Turn on the spectrophotometer and allow 15 min for warm up of the instrument prior to use. Use the wavelength knob to set the desired wavelength. With the sample cover closed, use the zero control to adjust the meter recorder to "0" on the % transmittance scale. Insert a clean cuvette containing the blank solution into the sample holder. The amount of solution placed in the cuvette is usually about 2/3 of the total volume of the cuvette. Close the cover and use the light control knob to set the meter recorder to "0" on the absorbance scale (100% transmission). Remove the blank cuvette, insert a cuvette holding the sample solution and close the cover. Read and record the absorbance. Remove the sample tube, readjust the zero, and recalibrate if necessary before checking the next sample. Carefully clean and store cuvettes for later use.

1.2.3 Requirements of spectrophotometric measurement

When use a spectrophotometric measurements one must understand that the absorption is produced by the particular absorbing substances (specific absorbance), but the solvent and substances in the reagents (nonspecific absorbance). The assay must include the following solutions:

(1) Blank solution/reference solution A proper blank solution contains none of the assayed substance, but all other chemicals in the test or standard solution and undergoes the same stages as the standard and test solution. This solution will help to exclude the absorption due to reagents (nonspecific absorbance).

(2) Standard solution It contains all the reagents of test and blank but also includes a solution of known concentration of the substance which is going to be determined in the test solution. It helps to correlate the absorption with the concentration of the concerned substance.

(3) Test solution/ sample solution/ determined solution It contains all the reagents present in the blank and standard and undergoes the same steps, but an unknown quantity of the

concerned substance.

1.3 Aplications of Spectrophotometry

1.3.1 Qualitative analysis

Spectrophotometry can be a very useful technique for identifying unknown compounds. Absorption spectra of a pure unknown compound and a standard known compound can be generated by measuring the absorbance at a variety of wavelengths. The shape of the spectra, λ_{max} (wavelength of maximal absorption) and ε (molar extinction coefficient) can be compared to identify the property of the unknown compound. If the two spectra are completely consistent, the sample and standard compounds can be tentatively determined to have the same chromophore group, and may be the same compound. Some substances with the same chromophore group but different molecular structure may also generate the same absorption spectra, but their absorption coefficient is different. If the absorption spectra, λ_{max} and ε are complete same, they are the same material. However, for really precise qualitative analysis other assays are required.

1.3.2 Quantitative analysis (determining the unknown concentration)

1.3.2.1 Standard addition method

The experimental approach exploits Lambert-Beer's Law, $A = KLC$, which predicts a linear relationship between the absorbance of the solution and the concentration of the analyte on the condition that K and L are constant. Prepare the test solution, the standard solution and the blank solution. All the solutions undergo the same treatment and their absorbances are measured under the same experimental conditions. Since the same absorbing substance, wavelength of light, temperature and length of the path of light, the K and L of the test and standard solutions are same. Let the concentration of the test unknown solution and standard solution is C_u and C_s, and the absorbance of unknown solution and standard solution is A_u and A_s, respectivly. $A_u = KLC_u$ and $A_s = KLC_s$, then $KLC_u/KLC_s = A_u/A_s$. S_o, C_u can be calculated by the formula as following:

$$C_u = \frac{A_u}{A_s} \times C_s$$

1.3.2.2 Calibration curve method

Calibration curve (also called working curve) shows how absorbance changes with the concentration of a solution. The curves are constructed by measuring the absorbance from a series of standards of known concentration and used not only to determine the concentration of an unknown sample but also to calibrate the linearity of an analytical instrument. A series of

standard solutions are prepared in calibration curve method then the absorbances are measured and used to prepare a calibration curve (standard curve), which is a plot of absorbance *vs* concentration. The absorbance of the unknown solution is detected and then used to determine the concentration of the compound in the test solution in conjunction with the calibration curve.

As a perfect calibration curve, assays should normally be performed in duplicate at least while preparing standard curve and only the mean should be plotted. There must be at least five points while plotting calibration curve. The points on the calibration curve should yield a straight line. The best straight line should be drawn through the points, the origin or other points. The calibration curve may vary in different batches of reagents and hence calibration curve will be done in each. Calibration curves should never be extrapolated beyond the highest absorbance value measured. If *A* is outside the range of the curve, the sample should be diluted or concentrated.

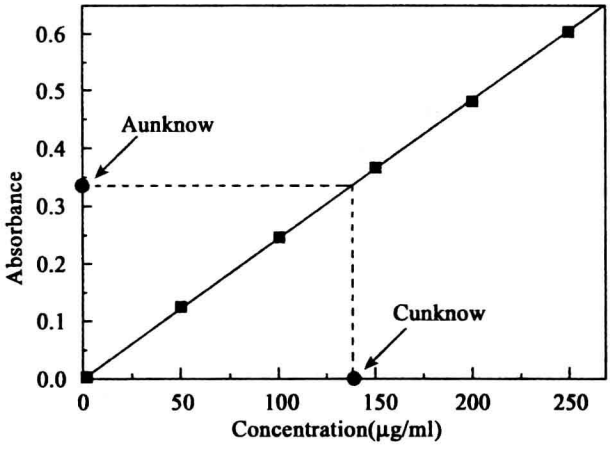

Fig. 1-4 Determining an unknown solution by calibration curve