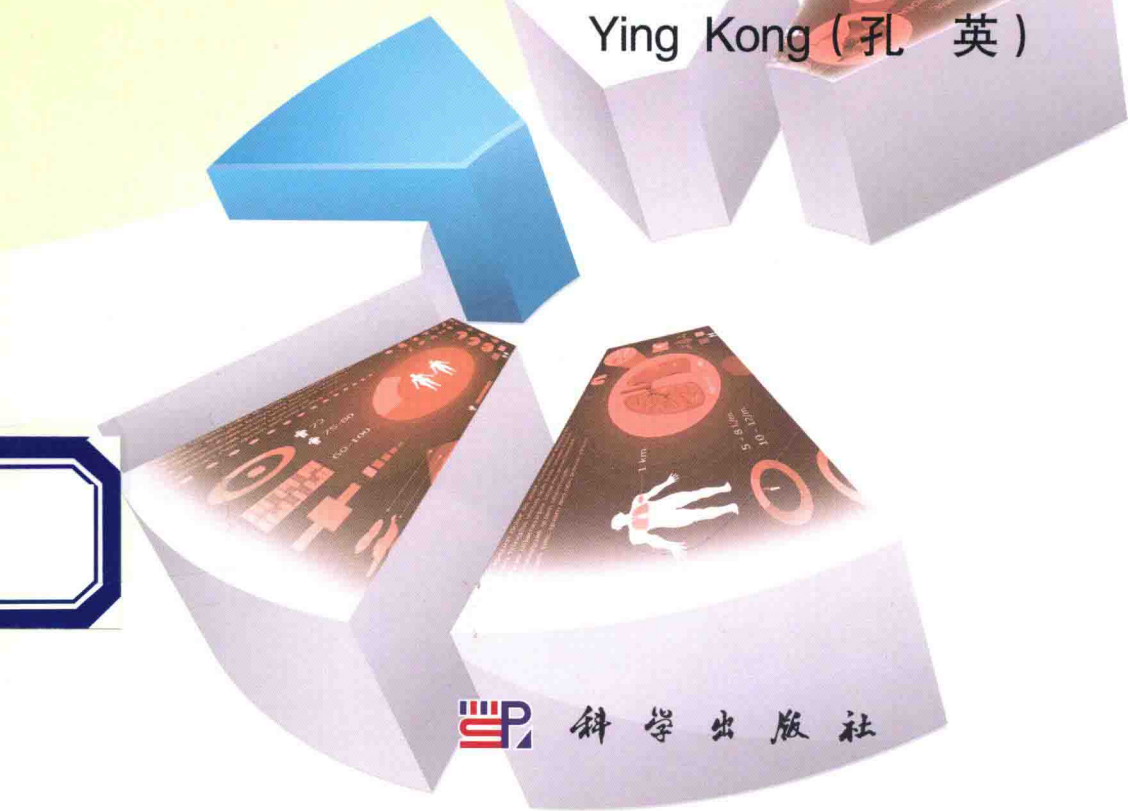
中国科学院教材建设专家委员会规划教材
全国高等医药院校规划教材

BIOCHEMISTRY AND MOLECULAR BIOLOGY LABORATORY COURSE

生物化学与分子生物学实验教程

（英文版）

Chief Editors Yuefei Xu（徐跃飞）
Yuxiang Tian（田余祥）
Ying Kong（孔　英）



科学出版社

中国科学院教材建设专家委员会规划教材
全国高等医药院校规划教材

BIOCHEMISTRY AND MOLECULAR BIOLOGY LABORATORY COURSE

生物化学与分子生物学实验教程

(英文版)

Chief Editors(主编)：Yuefei Xu(徐跃飞) Yuxiang Tian(田余祥) Ying Kong(孔　英)
Associate Editors（副主编）：Wenli Zhang(张文利)　Jianhui Fan(樊建慧)
Contributors(编委)：

Yuefei Xu(徐跃飞)	Wenli Zhang(张文利)
Jianhui Fan(樊建慧)	Yuxiang Tian(田余祥)
Ying Kong(孔　英)	Wenyong Ding(丁文勇)
Fan Yang(杨　帆)	Keli Ma(马克里)
Xuesong Yang(杨雪松)	Feng Ren(任　凤)
Qiu Yan(燕　秋)	Yufang Ma(马郁芳)
Ying Gao(高　颖)	Lin Lin(林　琳)
Shuai Liu(刘　帅)	Shujing Wang(汪淑晶)

科学出版社
北　京

内 容 简 介

本书针对生物化学教学及研究中涉及的三类主要的生物分子——蛋白质、酶和核酸，根据生物大分子的理伦性质，结合实用的电泳技术、层次技术、离心技术、分子克隆技术等，综合系统地介绍生物大分子的分离、纯化、含量测定、分析鉴定和应用，并结合教学中物质代谢、酶促反应动力学的系统分析，设计实验并融入相关实验技术和研究新进展，使学生在实验教学中掌握知识、技能的同时，体会对科研方法的选择与评价。

本教材内容新颖、科学、实用性强，可作为高等医学院校本科学生、留学生及研究生教材使用，也可供有关的教学及科研人员参考。

图书在版编目（CIP）数据

生物化学与分子生物学实验教程=Biochemistry and molecular biology laboratory course：英文 / 徐跃飞，田余祥，孔英主编. —北京：科学出版社，2014.9

中国科学院教材建设专家委员会规划教材·全国高等医药院校规划教材

ISBN 978-7-03-041896-8

Ⅰ. ①生… Ⅱ. ①徐… ②田… ③孔… Ⅲ. ①生物化学-实验-医学院校-教材-英文②分子生物学-实验-医学院校-教材-英文 Ⅳ. ①Q5-33 ②Q7-33

中国版本图书馆 CIP 数据核字(2014)第 215346 号

责任编辑：周万灏　王　颖 / 责任校对：鲁　素
责任印制：肖　兴 / 封面设计：范璧合

科学出版社 出版
北京东黄城根北街 16 号
邮政编码：100717
http://www.sciencep.com
骏杰印刷厂 印刷
科学出版社发行　各地新华书店经销
*
2014 年 9 月第　一　版　　开本：787×1092　1/16
2014 年 9 月第一次印刷　　印张：7
字数：151 000
定价：28.00 元
（如有印装质量问题，我社负责调换）

Preface

The achievements and developments in biochemistry and molecular biology are based on various scientific experiments. Without a basic knowledge of the principle and procedure of these experiments, one cannot profoundly comprehend the contents in biochemistry and molecular biology. It is essential for the students to understand and master the techniques and methods commonly used in this discipline. The aim of the teaching on experimental biochemistry and molecular biology is to train the students ability to think and resolve problems on their own, and to develop their initiative and creativity.

According to the arrangement for our experimental teaching, this manual is divided into two parts: Part Ⅰ and Part Ⅱ. The basic principles of some techniques, such as spectrophotometry, electrophoresis, chromatography, centrifugation, genetic engineering, etc., are mainly described in Part Ⅰ. Part Ⅱ consists of 25 experiments, each of which provides a detailed procedure for students to practice. Through the experimental teaching, we hope that students can obtain the basal skill straining of biochemistry and molecular biology, and deeply understand and master the biochemical theory .

This manual is not only applicable both to the undergraduates and graduates, but also to researchers in this field. Sincerely, we look forward to receiving more suggestions and comments from the students and teachers who use it.

Yuefei Xu, Yuxiang Tian,Ying Kong

April ,2014

前　言

生物化学与分子生物学的成就和发展都是建立在各种科学实验基础之上，对于这些实验的原理和步骤没有一个基本的了解，人们就不能更好地解读生物化学与分子生物学的内容。了解和掌握生物化学与分子生物学通常使用的技术和方法对于学生是十分必要的。生物化学与分子生物学实验教学的目的就是要训练学生独立思考问题和解决问题的能力，以及培育他们的主动性和创造性。

根据生物化学与分子生物学实验教学的安排，本书分为两大部分：第一部分主要阐述某些技术的原理，如分光光度技术、电泳技术、层析技术、离心技术和基因工程等。第二部分由 25 个具体的实验组成，每个实验内容都为学生提供一个详细的操作方案。我们希望通过本教材的实验教学，使学生能受到生化和分子生物学实验技术操作技能的基本训练，并有助于加深对生化理论的理解和掌握。

本书不仅适用于本科生和研究生，也适用于研究人员。我们真心地接受来自于使用本书的学生和教师的批评和意见。

徐跃飞　田余祥　孔　英

2014 年 4 月

Contents

Part 1 Basic techniques of biochemistry and molecular biology

Chapter 1 Basic techniques of preparation of biomacromolecules 1

Section 1 Salting out 2

Section 2 Dialysis and ultrafiltration 3

Chapter 2 Spectrophotometry 5

Section 1 Basic principles 5

Section 2 The basic construction of spectrophotometers 7

Section 3 Some home-made spectrophotometers 9

Chapter 3 Chromatography 12

Section 1 Adsorption chromatography 12

Section 2 Partition chromatography 12

Section 3 Ion-exchange chromatography 14

Section 4 Gel filtration 17

Section 5 Affinity chromatography 18

Chapter 4 Electrophoretic techniques 20

Section 1 Basic principles 20

Section 2 Several types of commonly used electrophoresis 23

Chapter 5 Centrifugation techniques 29

Section 1 Basic principle 29

Section 2 General description of centrifugation 30

Chapter 6 Genetic engineering and analytical technology of gene expression 34

Section 1 Genetic engineering 34

Section 2 Polymerase chain reaction 40

Section 3 Nucleic acid hybridization 45

Section 4 DNA sequencing technology 46

Part 2 Experiments of the biochemistry and molecular biology

Chapter 7 Analysis of purification and physicochemical properties of proteins 49

Experiment 1 Colorimetric methods for protein determination 49

Experiment 2 Isolation and purification of serum γ-globulin 51

Experiment 3 Cellulose acetate membrane electrophoresis of serum proteins 53

Experiment 4 Determination of relative molecular mass of protein in SDS-PAGE 55

Chapter 8 Kinetics of enzyme-catalysed reactions ····· 58

Experiment 1 The specificity of enzyme and effect of temperature, pH on the enzyme Activity ····· 58

Experiment 2 Isolation of alkaline phosphatase and determination of its specific activity 60

Experiment 3 Effects of substrates concentration of enzymatic activity: determination of K_m of alkaline phosphatase ····· 64

Experiment 4 Inhibition effect of phosphate on alkaline phosphatase ····· 66

Experiment 5 Analysis of lactic dehydrogenase isoenzyme ····· 67

Chapter 9 Substance metabolism ····· 70

Experiment 1 Extraction and identification of liver glycogen ····· 70

Experiment 2 Determination of alanine transaminase activity (Reitman-Frankels'method) ····· 71

Experiment 3 Enzyme catalyzed transamination of amino acids detected by paper chromatography ····· 73

Chapter 10 Basic experiment of molecular biology ····· 76

Experiment 1 Preparation of the genomic DNA in the eukaryotic cells ····· 76

Experiment 2 Determination of the concentration and purity of DNA by spectroscopy ····· 77

Experiment 3 Agarose gel electrophoresis of nucleic acids ····· 77

Experiment 4 Polymerase chain reaction (PCR) ····· 79

Experiment 5 Isolation and purification of the DNA fragments ····· 80

Experiment 6 DNA ligation—constructing recombinant plasmids ····· 82

Experiment 7 Preparation of *E.coli* competent cells ····· 83

Experiment 8 Transformation of Ca^{2+} competent cells ····· 84

Experiment 9 The screening of recombinant DNA ····· 85

Experiment 10 Minipreparation of plasmid DNA ····· 86

Experiment 11 Digestion of plasmid DNA with restriction endonucleases ····· 89

Experiment 12 Southern blot ····· 90

Experiment 13 Expression of exogenous gene in prokaryotic cells ····· 93

References ····· 95

Appendix ····· 96

Appendix Ⅰ The calculation table of ammonium sulfate saturation ····· 96

Appendix Ⅱ Common buffer solution preparation ····· 96

Appendix Ⅲ The relative molecular mass and pI of protein ····· 100

Appendix Ⅳ Common data of chromatography ····· 101

Appendix Ⅴ The conversion between centrifugation speed of centrifugation force ····· 103

Part 1 Basic techniques of biochemistry and molecular biology

Chapter 1 Basic techniques of preparation of biomacromolecules

The process of preparation of biomacromolecules (BMM) includes selection of starting raw materials, disruption of cells and isolation of organelles, extraction, isolation and purification of BMM, and also concentration, drying and storage of them. The preparation of pure BMM is a tough and time-consuming task, which needs scrupulous care in every step to preserve the biological activity during the whole process. The selection methods used in the preparation of BMM depend upon the physical and chemical properties of BMM (Table 1-1) including size, shape, solubility, charges, *etc*. BMM with different structures and physical properties may be separated and purified by different methods.

Table 1-1 Methods of isolation and purification of BMM

Basis of separation	Methods used
Molecular size and shape	Differential centrifugation, ultrafiltration, molecular seive,dialysis
Solubility	Salting out, extraction by solvents, partition chromatography,crystallization
Charges	Electrophoresis, electroosmosis, isoelectric focusing electrophoresis, ion exchange chromatography, adsorption chromatography
Specific biologic function	Affinity chromatography

In order to assess the efficiency of each step in the process of isolation and purification, an assay method of the BMM intended to be isolated and purified should be established prior to the isolation process. With the assay method, the BMM can be traced in the separation process, and the purity and the yield (or recovery) can be calculated. The specific activity of the product obtained in each step should be calculated by dividing the total activity by the total amount of proteins present. Also the percentage recovery of the activity should also be calculated by dividing the total activity obtained in each step by the total activity of the first step, and then multiply by 100. Table 1-2 represents an example of such isolation and calculations, in which the enzyme is isocitrate dehydrogenase (ICD) from pig liver. From Table 1-2, we know that ICD has a 570-fold purification, and the yield is 25.5 %.

Table 1-2 Purification of isocitrate dehydrogenase from pig liver

Procedure	Vol (ml)	Enzyme (U/ml)	Total units	Protein (mg/ml)	Total protein (mg)	Spec'act (U/mg)	Yield (%)	Puri'n (fold)
Homogenate	7.00	2.85	19.95	35.50	248.50	0.080	100	1.0
$CHCl_3$ extract	5.00	3.60	20.88	19.20	111.40	0.187	105	2.34
37.5 % – 55 % Salting-out	1.50	11.25	16.87	21.40	32.10	0.525	84.5	6.56
DEAE-cellulose	2.39	3.32	9.09	1.00	2.38	3.82	45.5	47.7
Ca-phos eluate	0.45	15.05	6.77	0.90	0.41	16.70	34	209
Gel filtration	0.52	9.80	5.09	0.22	0.11	45.60	25.5	570

In the following, brief discussions will be given to the basic techniques used in the process of isolation and purification of proteins, the most frequently encountered BMM.

Section 1 Salting out

This method is the earliest one used in the purification of proteins and enzymes. It is still used extensively now. The action of salt when in high concentration is to cause dehydration of the hydration layer of protein molecules, thus making the solubility of proteins decrease and, in turn, precipitate (salting out). Different proteins can be precipitated in different salt concentrations. On the contrary, the increase of solubility of proteins in low salt concentration is called "salting in".

During salting out, the relationship between solubility of proteins and the ionic strength (Chapter 4) of the solution can be expressed as follows:

$$\log\frac{S}{S_0} = -K_s \cdot I$$

Where S_0 is the solubility of protein in pure water, I is the ionic strength of salt solution, S is the solubility of protein in solution with ionic strength I, K_s is the salting out constant. In the above equation, when temperature and pH keep constant, S_0 is a parameter determined only by the nature of the protein. Therefore, under constant temperature and pH, S_0 of the given protein is a constant.

$$\text{Let} \quad \log S_0 = \beta$$

$$\text{Then} \quad \log S = \beta - K_s \cdot I$$

The salting out constant K_s is primarily determined by the nature of the salt, e.g, valence and average radius of the ion, and is also determined by the nature of the protein. Different proteins have different K_s values in a given salt solution, the higher the K_s value is, the better salting out result will be obtained. From the above equation, one could see that different proteins will have different β and K_s values at a given temperature and pH environment. The method by which ionic strength is varied to achieve salting out of different proteins is called "K_s salt fractionation". If the ionic strength is kept constant, the values of β might be changed by varying temperature and pH to achieve salting out of different proteins. This is called the method of "β salt fractionation".

The following conditions should be considered when proteins are purified by using salting out method.

1. Salt species Commonly used in the salting out of proteins are neutral salts including ammonium sulfate, magnesium sulfate, sodium sulfate, sodium chloride and sodium phosphate. The most extensively used one is ammonium sulfate, which has the following advantages:

(1) High solubility. At 25°C, the solubility of ammonium sulfate could be more than 4.1mol/L (541 g/L). Each liter of water can solubilize about 767 g of ammonium sulfate. In such a wide range of salt concentrations, many proteins and enzymes could be precipitated by salting out method.

(2) Low temperature coefficient. Temperature exerts little influence on the solubility of ammonium sulfate, e.g, at 0°C, its solubility could still be 3.9 mol/L (515 g/L) and each liter of water can solubilize 676 g. So it is advisable to use ammonium sulfate to purify enzymes at low temperatures.

(3) Less detrimental to proteins. Ammonium sulfate very seldom causes proteins denaturation, on the contrary, it can, to certain degrees, protect the activity of enzymes.

(4) Low price and readily available. The drawback of ammonium sulfate is that the

ammonium ions will interfere with the biuret reaction, thus renders the qualitative analysis of proteins difficult. Sometimes ammonium sulfate may contain contaminated heavy metal ions which will inactivate some enzymes. In such conditions the salt has to be recrystallized once or twice or more times to remove the heavy metal ions.

2. Salt concentration The separation of proteins by salt fractionation is achieved by changing the salt concentration. The salt concentration should accurately be raised high enough to precipitate different proteins. The concentration of salt is often expressed by the degree of saturation. The degree of saturation of saturated solution is 100 %. The amount of ammonium sulfate added to a solution containing ammonium sulfate with a known saturation S_1 to raise the degree of saturation to S_2 can be calculated or be determined by consulting to a pre-established table.

3. pH value As mentioned above, the value of β is closely related to pH value. When the pH of the solution reaches the pI of a given protein, β has its minimum value, and the solubility of that protein also reaches its minimum and it is most easily precipitated from the solution. Therefore, it is necessary to have the pH of the solution be adjusted nearest to the pI value of the protein in salting out.

4. Temperature The value of β is less influenced by temperature changes than by pH changes. So constancy in temperature is not strictly needed. Low temperature is preferred to avoid denaturation or hydrolysis of proteins.

5. Protein concentration The higher the concentration of protein in the solution is, the lower the salt saturation needed during salting out will be. But with too high protein concentration, the contaminant protein is liable to co-precipitate, thus the purity will be affected.

Section 2 Dialysis and ultrafiltration

1.2.1 Dialysis

Dialysis is a method used to achieve separation of BMM from small molecules. It is based on the capability of semi-permeable membrane to exclude BMM while the small molecules can pass through it. During dialysis, the solution containing BMM is put into the dialysis bag (or tube), the bag is being tied by a string or rubber band, and then immersed into distilled water in a large con- tainer. By stirring with a magnetic stirrer or other means (stirring device), the water in the container is made to flow continuously to assist diffusion. After certain period, the BMM still remains in the bag, which the small molecules of salt will go pass the semi- permeable mem- brane into the distilled water (Figure1-1). The concen- tration of salt on both sides of the membrane will reach equilibrium. Several changes of the dialysate outside the bag will bring the salt concentration in the bag to negligible level, thus desalting can be achieved.

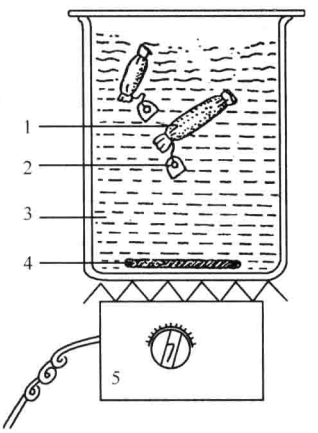


Figure 1-1 Diagrammatic representation of dialysis.
1. dialysis bag containing the solution to be dialyzed. 2. label. 3. water or buffer solution. 4. stirring bar. 5.magnetic stirrer

Desalting dialysis is the most extensively used

method, while equilibrium dialysis is also often used. The latter is done by placing the bag containing BMM and salt into a solution containing salt (or buffer) of another concentration. By dialysis, the concentration of salt or pH on both sides of the membrane becomes equal, thus the change of salt concentration can be achieved.

Solutions of BMM may be concentrated by dialysis against a high molecular weight non-permeating water-gathering polymer compound. This is called "reverse dialysis". The polymers which could be used in reverse dialysis include polyethylene glycol (Carbowax, PEG), polyvinylpyrrolidone (PVP), dextran, *etc*. In some instances, sucrose may also be used.

There are many semi-permeable membranes which can be used in dialysis, such as cellophane, collodion, membrane of animal origin and parchment, *etc*.

1.2.2 Ultrafiltration

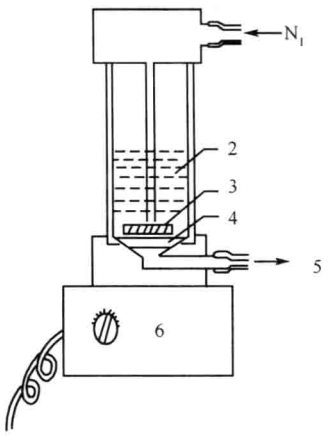


Figure 1-2 Diagrammatic representation of ultrafiltration
1.inlet for N_2 at a given high pressure. 2.BMM retained in the ultrafiltration chamber. 3. stirring rod. 4. ultrafilter. 5. outlet for water and small molecules. 6.magnetic stirrer

Ultrafiltration is potentially the most useful technique, particularly since membranes of different porosities are now available commercially. In ultrafiltration, the protein or enzyme solution is fed into a cell fitted with a membrane, which will retain the protein chosen while being permeable to solvent and to small molecules. Positive pressure applied to the solution, or negative pressure to the solvent collecting chamber, causes flow across the membrane (Figure1-2). Quite large volumes can be reduced to a few ml in the course of one or two hours. For retaining and concentrating proteins of low molecular weight, low porosity ultrafilters must obviously be used, but for larger proteins, higher porosity filters will be more convenient because the solvent flow rate is likely to be faster. The successive use of ultrafilters include using the one with high porosity first allowing a given enzyme to pass through, and then the other one with low porosity to retain the enzyme, which provides a technique for purification as well as concentration.

1.2.3 Concentration under reduced pressure and lyophilization

In concentrating and drying of samples, because of the liability of BMM, evaporation at high temperatures is not recommended.

Therefore, concentration under reduced pressure and freeze-drying (lyophilization) has been widely used nowadays. Rotary evaporator is one device used in concentration under reduced pressure. In freeze-drying, the solution to be dried is first frozen at low temperature, and then negative pressure is applied to the chamber where the frozen sample is laid. After sublimation of frozen solvent, dry samples of BMM will be obtained. The lyophilized product will retain most of the natural properties of the BMM which includes the biological function. Also they are porous, readily soluble, and easy to store and to be used.

(Qiu Yan, Xuesong Yang)

Chapter 2 Spectrophotometry

Section 1 Basic principles

When a beam of natural light is passed through a solution containing a colored compound, certain ranges (wavelengths) of light are selectively absorbed. Different compounds absorb different wavelengths of light according to their different molecular structures. Every compound has its own specific absorption spectrum. So the plots of absorption spectrum can be used to characterize compounds. For example, riboflavin has one absorption maximum in the entire visible region (400–700 nm), *i.e.*, 450 nm, while in the ultraviolet range, two absorption peaks (which are 260 and 370 nm respectively) exist (Figure2-1). The absorption spectrum can explain the yellow color of riboflavin, *i.e.*, the wavelength of the unabsorbed transmitted light lies in the yellow region.

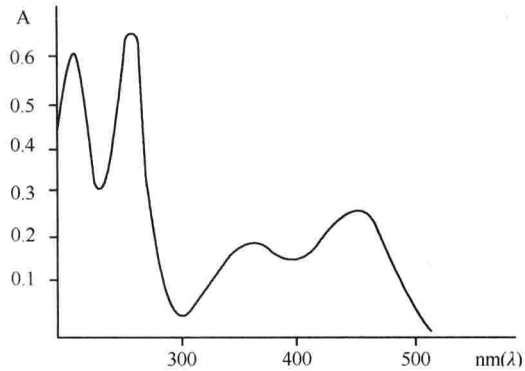


Figure 2-1 Absorption spectrum of riboflavin (22 μmol/L in 0.1 mol/L sodium phosphate, pH 7.06, in 1 cm light path)

Photometry involves the qualitative and quantitative use of absorption data obtained from compounds that absorb light in the ultraviolet (UV, 200–400 nm), visible, and near infrared (near IR, 700–900 nm) regions. Much of photometry is based on two formalized laws, *i.e.*, Lambert's law and Beer's law.

2.1.1 Lambert's law

If a beam of monochromatic light is passed through a solution, part of the light is absorbed by the solution. The intensity of transmitted light is weaker than that of the incident light, when the concentration of the solution is fixed, the thicker the light path is, the weaker the intensity of transmitted light will be (Figure 2-2).

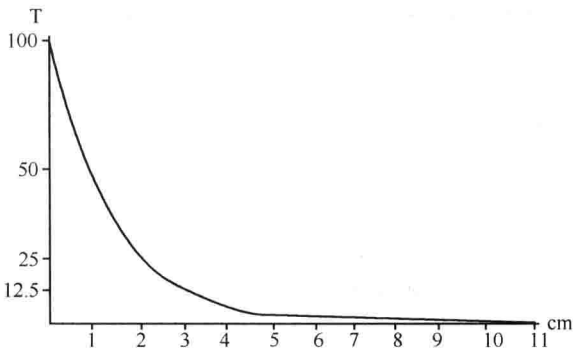


Figure 2-2 The relationship between the light absorption and the thickness of the solution

Let I_0=intensity of the incident light, I=intensity of transmitted light, L=length of light

path(thickness of the solution),then

$$\frac{-\mathrm{d}I}{\mathrm{d}L} \propto I, \qquad \frac{-\mathrm{d}I}{\mathrm{d}L} = aI, \quad \text{or} \quad \frac{\mathrm{d}I}{I} = -ad\ L \tag{2-1}$$

$$\int \frac{\mathrm{d}I}{I} = -\int ad\ L$$

$\ln I = -\alpha L + C$

If $I = 0$, i.e., $I = I_0$, $C = \ln I_0$ $\quad \ln I = -\alpha L + \ln I_0$

Thus $\quad \ln \frac{I_0}{I} = \alpha L \quad$ or $\quad \frac{I}{I_0} = \mathrm{e}^{-\alpha L}$ (2-2)

or $\quad \lg \frac{I_0}{I} = K_1 L \quad \left(K = \frac{\alpha}{2.303}\right), \quad \frac{I}{I_0} = 10^{-K_1 L}$

α is the absorption coefficient, and K_1 is the extinction coefficient which is influenced by the wavelength of light, the nature of the solvent and the concentration of the solute in the solution. As shown in equation(2-2), the diminution of the intensity of incident light(I/I_0), as the light is passed through a solution, is not simply in proportion to the thickness of the solution, but has the relationship of exponential function with the thickness of the solution. This is the Lambert's law.

2.1.2 Beer's law

If a beam of monochromatic light is passed through a solution with fixed thickness, the higher the concentration is, the weaker the intensity of the transmitted light. The relationship between those is similar to Lambert's law which is shown as follows.

$$\lg \frac{I_0}{I} = K_2 C, \text{ or } \quad \frac{I}{I_0} = 10^{-K_2 C} \tag{2-3}$$

Where C=concentration of solution, K_2 is a constant influenced by the wavelength of light, the nature of solvent and the thickness of the solution. Equation (2-3) which expresses the relationship between light intensity and the concentration of solution is termed as Beer's law.

Even though all solutions conform to Lambert's law, but not all solutions conform to Beer's law. The reason is that some compounds at various concentrations may have different absorption spectra and different colors. For example:

(1) Some colored compounds can be dissociated in solutions into ions which have different colors from their molecular forms.

(2) Chelate can be formed in high concentration of some solutions. The absorption spectra of these chelates differ from their original molecules. For example, cobalt chloride shows roseate at low concentrations and blue at high concentrations

$$CoCl_2 + CoCl_2 \rightarrow Co^{2+}\ (CoCl_4)^{2-}$$

Roseate Blue

(3) Colors of some colored compounds can also be changed by hydrogen ions and by some electrolytes.

2.1.3 Lambert-Beer's law and its application

Lambert's and Beer's laws can be combined together as follows:

$$\lg \frac{I_0}{I} = KCL, \quad \text{或} \quad \frac{I}{I_0} = 10^{-KCL} \tag{2-4}$$

Here I/I_0 is termed transmittance (T), $-\lg I/I_0$ is termed absorbance (The historic term optical density, O.D., should not be used), the latter expresses the extent of absorption by solution.

$$A\ (D) = -\lg \frac{I}{I_0} = -\lg T = KCL$$

Where K is termed extinction coefficient (E) representing the ability of light absorption of solution.

It should be remembered that a K value is given for a particular wavelength and solvent and that another K value applies at a different wavelength or with another solvent.

As known from equation (2-4), if solvent and wavelength are fixed (extinction coefficient is fixed), the absorbance of a solution is proportional to its concentration.

$$\frac{A_1}{A_2} = \frac{C_1}{C_2} \quad \text{or} \quad C_1 = \frac{A_1}{A_2} \times C_2 \tag{2-5}$$

Where C_2=concentration of standard solution, C_1=concentration of unknown solution which can be calculated after measuring the absorbance of standard and unknown solutions respectively.

In practice, a series of standard solutions are spectrophotometricly measured and a plot of absorbance against concentrations of standard solutions is made. Using this standard curve, the concentration of an unknown solution can be found according to the absorbance.

As known from equation (2-4), if extinction coefficient and the thickness of the solution are known, the concentration of the solution could be mathematically obtained from the measured absorbance. There are two ways which are employed to express extinction coefficient:

(1) Molar extinction coefficient:The molar extinction coefficient, $E_{1cm}^{1mol/L}$ (often written as ε), is numerically equivalent to the absorbance ($\lg I/I_0$) of a 1mol/L (solution of the substance, with a light path of 1 cm. Because absorbance ($\lg I_0/I$) is dimensionless, ε is expressed in terms of liters/(moles×length), or liters $moles^{-1}cm^{-1}$.

(2) Percentage extinction coefficient:Percentage extinction coefficient ($E_{1cm}^{1mol/L}$) is numerically equivalent to the absorbance of 1% of the substance with a light path of 1 cm.

The equations for calculating concentration of substance are shown as follows:

$$C=A/\ E_{1cm}^{1mol/L} \text{ (unit of concentration is g \%).}$$

$$C=A/\varepsilon \text{ (unit of concentration is mol/L).}$$

For example, if the absorbance of a protein solution at λ_{278} nm is 0.520, the thickness of the cuvette is 1.000 cm, the known $E_{1cm}^{1\%}$ 278=5.10, the concentration of the protein solution is:

$$C=A/\ E_{1cm}^{1mol/L}=0.520/5.10=0.102\ (g\%).$$

Section 2 The basic construction of spectrophotometers

Photometers, colorimeters, and spectrophotometers employ the common basic components including light source, wavelength selector (monochromators), slit, sample tube (cuvette) and light-detecting system (Figure 2-3).

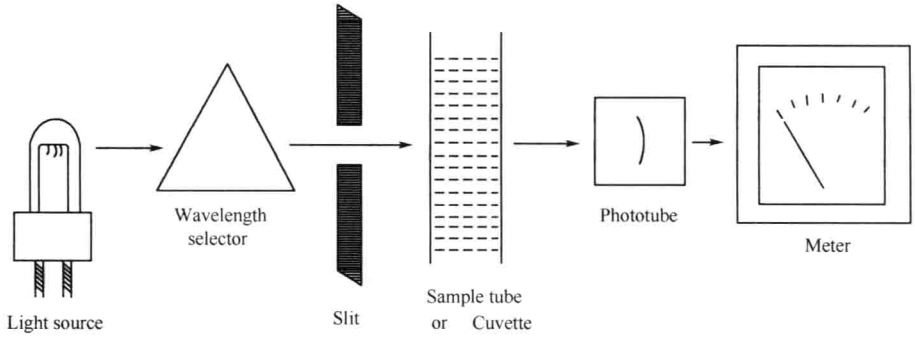


Figure 2-3 Operational diagram of a photometer or spectrophotometer

2.2.1 Light source

The light source must be capable of emitting a steady amount of sufficient energy in a wide wavelength ranges. Most photometers employ a constant voltage-regulated tungsten lamp for spectral analyses in the range of 340–900 nm. More sophisticated spectrophotometers, which also have capability for analysis in the UV range, employ an additional constant voltage-stabilized hydrogen lamp that emits light in the range of 200–360 nm.

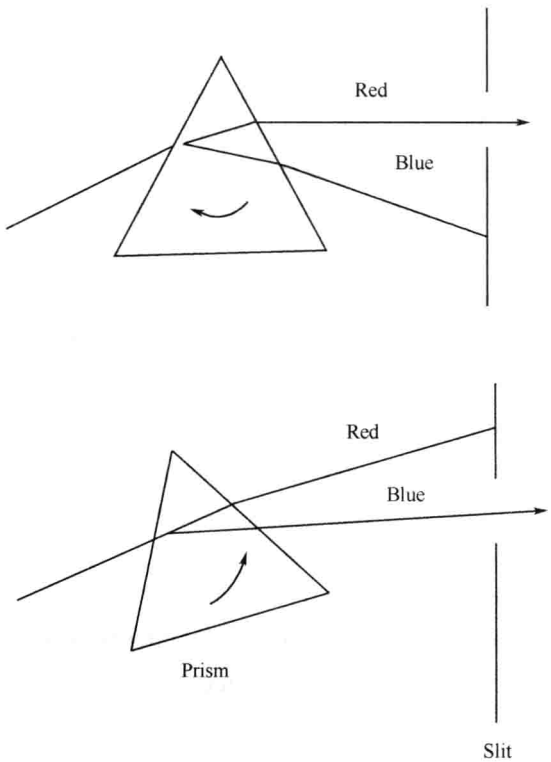


Figure 2-4 Generation of monochromatic light by the prism

2.2.2 Monochromators

Photometry requires assay of absorbance at given wavelength. Monochromators are employed to generate the desired wavelengths of light. Usually, it is not possible to have light representative of a single wavelength. Therefore, when we speak of monochromatic light, we usually mean a source that has its maximum emission at this wavelength and has progressively less energy at longer and shorter wavelengths. We must say that the greater the spectral purity of monochromatic light, the greater the sensitivity and resolution of measurements.

The simplest wavelength selectors are one or more absorption filters that screen out light above and below specific wavelengths. Many of the older colorimeters employ such filters because of their simplicity and low cost. However, these filters generally have broad transmission ranges, and therefore the resolution of absorption spectra is low. The modern monochromators containing a prism or a diffraction grating can overcome these defici- encies. Such monochromators generate relatively pure light at any wavelength over a wide range (Figure 2-4).

2.2.3 Slit

The intensity of light emitted through any filter or monochromators may be too intense or too weak for a light-sensing device to record. It is therefore necessary to be able to adjust the intensity of the incident light (I_0) by placing a pair of baffles in the light path to form a slit. The desired intensity of light can be obtained through adjusting the width of slit. Simple colorimeters often have a fixed slit, but more sophisticated spectrophotometers usually have a variable slit mechanism.

2.2.4 Cuvette

Sample cuvette is one of the most important components. Optical glass cuvettes are employed in visual range (400–700 nm) in assays, and quartz cuvettes are in UV range (200–400 nm). In order to keep the cuvette at very good working conditions, the optical surface of the cuvette must be protected from damage. Do not touch the surface with fingers and rough things. After assay, the cuvette must be rinsed with distilled water immediately, and do not remain any measuring solution in the cuvette, especially the solutions of proteins and nucleic acids.

2.2.5 Light detecting system

Selenium photovoltaic cell, vacuum phototube and photomultiplier tubes can be employed as the photosensitive instruments through which the light energy is turned into electric energy. The current induced is proportional, within a certain range, to the intensity of light incident upon the instrument.

Colorimeter uses selenium photovoltaic cell as the light receptor. The cell is limited by low sensitivity (it does not detect light of very low intensity) and insensitivity to wavelengths shorter than 270 nm and longer than 700 nm.

More accurate spectrophotometers usually use vacuum phototubes or photomultiplier tubes as the light detectors and there are some instruments to amplify the photocurrent for increasing sensitivity. Although the intensity of monochromatic light with very narrow range of wavelength is weaker than that with much wide range of wavelength, this weak monochromatic light also can be recorded by sensitive detecting system with amplifying instruments.

Section 3 Some home-made spectrophotometers

2.3.1 Spectrophotometer model 721

This is the spectrophotometer with vacuum phototube as light detecting system. The range of wavelength detected is visual. The natural light with a steady amount of sufficient energy is emitted by a tungsten lamp. As shown in Figure 2-5, a beam of light is reflected by a reflection mirror (semi-transparent) and passes through a slit, then the light is reflected to a prism by a collimating mirror and the reflex is diverged to give the light spectrum. The prism is turnable, connecting with a scale, and the desired wavelength can be selected by

turning the prism. The monochromatic light we want goes back through the same slit, and then penetrates the semi-transparent mirror mentioned above. A cuvette containing the colored solution to be measured is put in the light path. The light that passes through the cuvette goes to a vacuum phototube which transduces the light energy into electric energy. The current generated by the phototube is shown on the galvanometer. The wavelength range given by spectrophotometer model 721 is 360–800 nm, but only 410–710 nm is recommended for use.

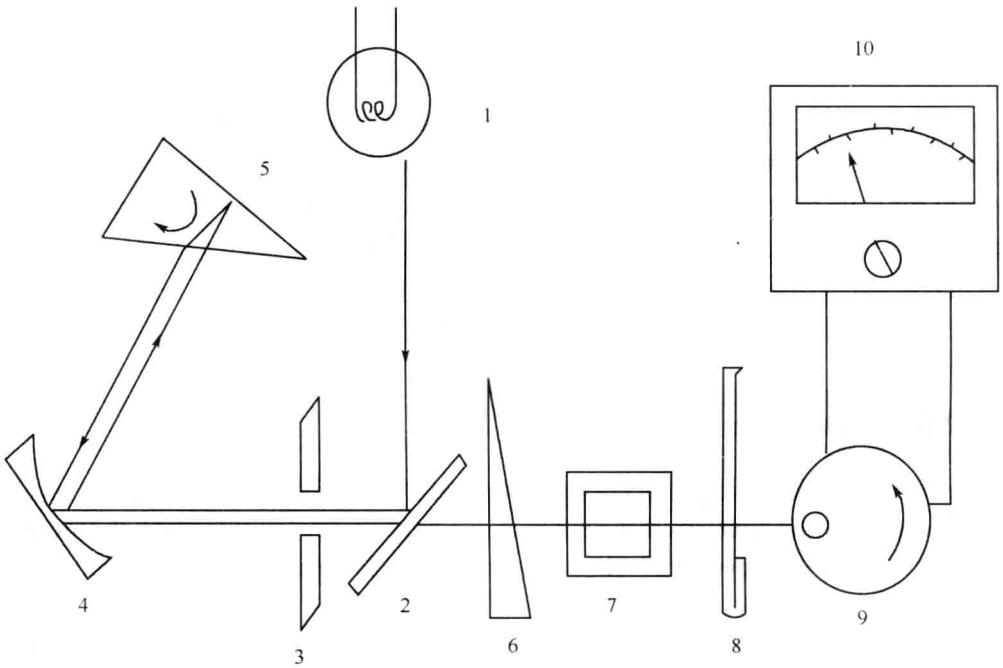


Figure 2-5 Diagram of light pathway of spectrophotometer model 721

1. light(tungsten lamp). 2. semi-transparent mirror. 3. slit. 4. collimating mirror. 5. prism. 6.light shutter. 7.cuvette. 8.light gate.9.vacuum phototube. 10.galvanometer

2.3.2 Spectrophometer model 754

This is the spectrophotometer with the capability of measuring in the ultraviolet, visible and infra-red regions (200–1000 nm). It can be used to plot absorption spectrum. The optical system is similar to model 721, but quarts-rather than glass-made prism as monochromator and two light sources (tungsten and hydrogen lamps) are used This instrument has a more com- plicated electric system (Figure 2-6). After being amplified by the amplifying system, photo- electric current is measured by a potentiometer. The advantage is that it has higher resolution power, because the divisions on the scale are larger. Transmittance and / or absorbance can be read directly from the dial.