Experimental Biochemistry

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Preface

"Progress in science is made by observation and experiment. This is particularly evident in biochemistry, where the remarkable advances of the past two decades have been made possible largely by the introduction of new methods and techniques." These words, which began the Preface of the Second Edition of *Experimental Biochemistry* (1977), by John M. Clark, Jr., and Robert L. Switzer, are equally appropriate as we complete the Third Edition.

Goals of the Third Edition. Although the foundations laid by twentieth century biochemists have proven to be quite solid, the more than twenty years since the previous edition was published have seen so many advances in both the fundamental understanding and the technology of biochemical research that extensive and continuous revision of the content of general biochemistry laboratory instruction is imperative. This edition has grown out of our efforts, and the efforts of our colleagues at the University of Illinois at Urbana-Champaign, to introduce useful new experimental techniques, tools, and materials into our own biochemistry laboratory instruction. The new and revised experimental exercises in this edition were developed in the Department of Biochemistry at the University of Illinois and have been extensively tested in typical instructional settings in our biochemistry laboratory course for advanced undergraduates and beginning graduate students. We are confident that the new edition can be used in a wide variety of settings for instruction in the most important techniques of modern biochemistry. Students without previous biochemical laboratory experience can develop mastery of the theory and practice necessary for advanced laboratory work or for their first experiences in biochemical research. Such education in biochemical techniques is even more important today than in the past. Developments in modern chemistry and biology have shown that biochemistry must today be regarded as a "root science." As science moves toward more integrated forms of investigation, virtually every student of biology and chemistry requires substantial formal training in modern biochemical laboratory techniques to be adequately prepared for a scientific career in the twenty-first century. It is our hope that *Experimental Biochemistry* Third Edition will provide a valuable tool for educators seeking to meet that goal.

Features of the Third Edition. As was true of previous editions of *Experimental Biochemistry*, this book attempts to provide a broad range of hands-on experiences with the most important and commonly used techniques of contemporary biochemistry. We have included exposure to all the major classes of biological molecules and topics of current biochemical and molecular biological research: proteins and enzymes, nucleic acids, lipids and membranes, carbohydrates, as well as metabolic systems, regulation, and the newly emerging field of biochemical information science. In no area of research have advances since 1977 been more extensive and revolutionary than in molecular biology. The section on nucleic acids and molecular biology has therefore been extensively revised. This book cannot be regarded as a comprehensive laboratory text for a molecular biology or molecular genetics course, however. This specialty is well served by a number of other texts.

Traditional Strengths Were Retained. In preparing this revision, we have attempted to strike

a balance between experiments using entirely new techniques and materials and revisions of more classical experiments from previous editions of *Experimental Biochemistry* that we find valuable for teaching and strengthening the fundamental principles of the discipline. Thus, experiments such as those in Section I, Experiments 5, 6, 7, 8, 12, 14, 19, and 20 present experimental exercises in "classical" biochemistry, which we believe still form an important part of a thorough training. These experiments have all been significantly revised to incorporate improvements in methods or materials, where applicable, and to improve clarity of presentation. Other experiments, which we felt were less useful or were outdated, have been omitted from the Third Edition.

Extensive New Material Has Been Incorporated. Many sections and experiments are entirely new in the Third Edition. These include Experiments 10, 15, and 16, an entirely new section on Immunochemical Techniques, which includes Experiments 17 and 18, Experiments 21, 22, 23, and 24 in the Nucleic Acids and Molecular Biology section, as well as Section VI on biochemical information science. All of these new experiments have been performed many times in our own laboratory classes. We are confident that they will provide reliable and stimulating educational experiences in all instructional settings.

Use of Radioisotopes in Experimental Biochemistry. Some reviewers have expressed concerns about the use of radioisotopes in some of the experiments in this text. Although it is true that alternative techniques are being developed that lessen the dependence of biochemical research on the use of radioisotopes, we firmly believe that radioisotopes will continue to be important in the foreseeable future, and that an adequately trained biochemist must be aware of these techniques and the power of radioisotopic techniques in biochemical research. The experiments in this text are designed to minimize the levels and exposure of students and staff to radioisotopes. They are completely safe for students and present no contamination hazards if the students are adequately supervised. Handling of significant amounts of radioisotopes is restricted to the teaching staff in the preparation of the experiments. In the same vein, we have attempted to identify clearly and forcefully in the text all instances where good laboratory practice requires special precautions and safety considerations.

Flexible Organization and Uses of the Contents. This book is intended as a text and laboratory manual for a one-semester laboratory course, but it is not realistic for the students in such a course to complete all 25 experiments. Rather, we expect that instructors will choose those portions of the text that suit their schedules, interests, and laboratory facilities. Some further thoughts about optimal and individualized uses of *Experimental Biochemistry* in the teaching laboratory are presented in the section entitled "To the Instructor" in the introductory chapter to Section I. Each of the six Sections is preceded by an extensive Introductory Chapter that reviews relevant background and fundamental biochemistry of a particular class of biological molecules, with a strong emphasis on experimental methodology. It has been our experience with previous Editions of *Experimental Biochemistry* that many instructors and students find these introductory chapters valuable even when they are performing lab exercises other than those in the text. Also, many students continue to use *Experimental Biochemistry* as a reference text long after completing their formal lab course. We certainly hope that the new edition will be even more valuable in these auxiliary uses.

Acknowledgments. The preparation of this laboratory manual was not solely the product of the labor of the two authors. We owe a great debt of gratitude to many colleagues and teaching assistants at the University of Illinois, who over the past twenty years have contributed ideas, techniques, and experience to the repeated revisions of our laboratory exercises. First, we acknowledge our former colleague, Professor John M. Clark, Jr., who retired from the University of Illinois in 1990; his many important contributions as author, and coauthor, respectively, of the First and Second Editions of *Experimental Biochemistry* continue to be found in many forms throughout the Third Edition. In addition, we gratefully acknowledge Professor David Kranz and Dr. Michael Saulmon for their major contributions to Section IV (Immunochemical Techniques) and Section VI (Information Science), respectively. Dr. Charles Matz contributed numerous improvements and refinements

during the period when he served as an instructor in Illinois' biochemistry laboratory course. We thank Professor Peter Imrey for preparing the section on statistics in the Introductory Chapter to Section I, Professor JoAnn Wise for her contributions to experiments in molecular biology, Dr. Kenneth Harlow for his assistance in developing the application of HPLC to amino acid analysis, Professor Evan Kantrowitz of Boston College for contributions to the modernization of Experiment 9, Dr. Charles Matz and Daryl Meling for assistance in development of Experiment 24, Christopher Wall for experimental testing of revised form of Experiment 9, and Kim Hines of the Pierce Chemical Co. for her expertise and contributions of reagents that were important in developing the chemiluminescent assay of Western blots. We are grateful to Professor Peter Orlean and Professor Michael Glaser for critical readings of the Introductory Chapter to Section III (Biomolecules and Biological Systems). Finally, we express our appreciation to our colleagues on other campuses who reviewed portions of our text prior to publication: Frank N. Chang, Temple University; Harry L. Price, University of Central Florida; Mike Panigot, Arkansas State University; Theodore Chase Jr., Cook College, Rutgers University; Larry Byers, Tulane University; Larry G. Ferren, Olivet Nazarene University; James H. Hageman, New Mexico State University; Nat Ramani, SUNY at Westbury; Joe Provost, Moorhead State University; and Robert Lindquist, San Francisco State University.

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About the Authors

Robert L. Switzer is Professor of Biochemistry at the University of Illinois at Urbana-Champaign, where he has been on the faculty since 1968. He holds a Ph.D. in Biochemistry from the University of California at Berkeley and was a postdoctoral fellow in the Laboratory of Biochemistry of the National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, from 1966 to 1968. His research centers on the regulation of enzyme activity and the control of gene expression by novel mechanisms in bacteria. He was a Guggenheim Fellow in 1975. Professor Switzer has had a career-long interest in biochemistry laboratory instruction and has twice received the School of Chemical Sciences Excellence in Teaching Award. He was a co-author with John M. Clark, Jr., of the Second Edition of *Experimental Biochemistry*.

Liam F. Garrity received his B.S. degree in Biochemistry from the University of Wisconsin-Madison in 1991 and his Ph.D. in Biochemistry from the University of Illinois at Urbana-Champaign in 1996. His graduate work focused on the regulation of kinase activity and the phosphoryl group transfer cascade governing chemotaxis in *Bacillus subtilis*. From 1996 to 1998 he served as a Visiting Lecturer in the Department of Biochemistry at the University of Illinois at Urbana-Champaign, where he supervised the biochemistry laboratory and lecture course for undergraduates and beginning graduate students. He received the Excellence in Teaching Award from the School of Chemical Sciences for his instruction in the general chemistry program while serving as a graduate student. Dr. Garrity is now with Pierce Chemical Company in Rockford, Illinois, which manufactures products for protein chemistry, immunochemistry, and molecular biology.

Contents

SECTION I

Basic Techniques of Experimental Biochemistry

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Introduction

Biochemistry is the chemistry of biological systems. Biological systems are complex, potentially involving a variety of organisms, tissues, cell types, subcellular organelles, and specific types of molecules. Consequently, biochemists must separate and simplify these systems to define and interpret the biochemical process under study. For example, biochemical studies on tissue slices or whole organisms are followed by studies on cellular systems. Populations of cells are disrupted, separated, and their subcellular organelles are studied. Biological molecules are studied in terms of their specific mechanisms of action. By dividing the system under study and elucidating the action of its component parts, it is possible to then define the function of a particular biological molecule or system with respect to the cell, tissue, and/or organism as a whole.

Biochemical approaches to the simplification and understanding of biological systems require two types of background. First, biochemists must be thoroughly skilled in the basic principles and techniques of chemistry, such as stoichiometry, photometry, organic chemistry, oxidation and reduction, chromatography, and kinetics. Second, biochemists must be familiar with the theories and principles of a wide variety of biological and physical disciplines often used in biochemical studies, such as genetics, radioisotope tracing, bacteriology, and electronics. This need reflects the biochemists' ready acceptance and use of theories and techniques from allied areas and disciplines.

It is not possible or appropriate for this book to summarize the many disciplines and principles used in biochemistry. However, students will find that a review of the basic principles and units used in the quantitative aspects of experimental biochemistry is quite useful. This section is intended to provide such a review. In addition, it is valuable for students to understand the methods often used in experimental biochemistry. Experiments 1 to 4 of this section deal specifically with these techniques: spectrophotometry, chromatography, radioisotope tracing, and electrophoresis. Finally, it is imperative that students understand the intricacies of data analysis. The final part of this Introduction discusses the principles underlying the basics of statistical analysis that are critical to the ability to determine the precision or error associated with quantitative data obtained in biochemical experiments.

Requirements for a Student of Experimental Biochemistry

This course is aimed at developing your interest in and understanding of modern biochemical and molecular biological experimentation. This goal necessitates a careful emphasis on the experimental design, necessary controls, and successful completion of a wide variety of experiments. This goal will require additional efforts if you are to benefit fully from *Experimental Biochemistry*. First, you should familiarize yourself with general background material concerning each experiment. Three elements have been incorporated into the text to aid you in this effort: (1) Each experiment

is preceded by a short introduction designed to aid you in understanding the various theories and techniques underlying the exercises. (2) The experiments are divided into sections that deal with a specific class of biological molecules. The introduction preceding each section will serve as a review to provide you with enough information to understand the experiments. This material is intended to reinforce and supplement the knowledge you have gained from biochemistry lecture courses and textbooks of general biochemistry, which you should review as needed. (3) Each experiment is followed by a set of exercises and related references that will allow you to further develop your interest and understanding of a particular method, technique, or topic.

Second, you must keep in mind that the ability to complete the experiments within allocated times requires you to be familiar with the protocol of the experiment *before the start of the laboratory session.* Each of the experiments contains a detailed, class tested, step-by-step protocol that will enable you to perform, analyze, and interpret the experiments on your own. Your success will depend on your ability to organize and understand the experimental procedures, making efficient use of your time.

Third, efficient use of *Experimental Biochemistry* requires that you perform and interpret many calculations *during the course of the laboratory sessions.* Specifically, laboratory work for introductory biochemistry, unlike many introductory laboratory courses, frequently requires you to use the results of one assay to prepare and perform additional assays. Thus, you will have to understand fully what you are doing at each step and why you are doing it.

Finally, it is *imperative* that you maintain a complete research notebook containing all your data, calculations, graphs, tables, results, and conclusions. Your notebook should be so clear and complete that *anyone* can quickly understand what was done and what results were obtained. Your instructor may provide additional specific instructions for your laboratory reports; the following suggestions may be helpful:

1. Use a large, bound notebook, preferably one with gridded pages. Such notebooks permit the direct construction of data tables and allow you to attach records of primary accessory data, such as computer-derived graphs, chromatograms, dried SDS-PAGE gels, and photographs.
2. Never record your data on separate sheets of paper. Rather, record all your data directly in your notebook. You may consider using one side of the notebook for raw data and calculations and the other side for results and interpretation.
3. All graphs and tables must be clearly and unambiguously labeled. Be particularly careful to specify units on the ordinate (y-axis) and abscissa (x-axis) of every graph.
4. The laboratory report for all experiments should include:
   a. a brief statement of purpose (why you are doing the experiment and what you wish to determine);
   b. a brief account of the theory and design of the experiment, including a summary or flow chart of the principal manipulative steps;
   c. the raw data;
   d. all calculations (if analysis requires a single, repetitive calculation, a sample calculation for one of a series is acceptable);
   e. results;
   f. conclusions and interpretations (the information that you can derive from the results of the experiment).

As stated earlier, all the experiments in this textbook have been class tested by hundreds of students. The experiments, therefore, show a high rate of success. Still, there may be times when your experimental results are not particularly useful, or when they yield unexpected results that require an explanation. If this is the case for a particular experiment, discuss in the results section of your laboratory report what may have gone wrong. Did you make an improper dilution of one of the reagents? Did you accidentally omit one of the experimental steps? In the conclusion section, discuss what you may have expected to see if the experiment had been successful. Your knowledge of the theory underlying the techniques, along with your understanding of the experimental protocol, should be sufficient to allow you to determine what type of data you may have obtained under ideal conditions. By doing this, you are likely to turn what appears to be a failed

experiment into a valuable learning opportunity. It is never sufficient to say, "the experiment did not work." Attempt to understand why a particular experiment may not have worked as expected.

Laboratory Safety

Experimental Biochemistry employs the use of potentially hazardous reagents. Strong acids, strong bases, volatile compounds, flammable compounds, mutagenic compounds, corrosive compounds, radioisotopes, electricity, and sharp objects are the tools of the biochemist. Like any other tool, these are hazardous only when handled improperly. At the beginning of each experimental protocol, we draw your attention to potential hazards that may be associated with a particular reagent you are about to use.

Safety goggles/glasses must be worn in the laboratory at all times. The main purpose of eye protection is to prevent chemical damage to the eye. Laboratory eye protection should also be shatterproof to protect against debris that would be produced from broken glass in the event of an accident. Although you may feel confident that you will not be the cause of such an accident, it is impossible to ensure that your laboratory partner or neighboring groups will not have accidents.

It is advised that you wear latex or vinyl exam gloves at all times in the laboratory. Even if a particular experiment does not require the use of hazardous chemicals, one can never be sure that those from a previous experiment have been properly disposed of. If volatile compounds are used, they should be stored under a fume hood at all times. If possible, students should work with these materials under the fume hood as well. The large amounts of materials that are often required for a laboratory group may soon fill the room with unpleasant and potentially hazardous vapors. This is particularily important if the reagent vapors are flammable (see Experiment 6) or radioactive (see Experiment 12).

Laboratory coats may be worn if desired. It is a good idea to wear them when working with radioisotopes, since very small quantities of a radioactive solution can carry a significant amount of activity. It is also a good idea to wash your hands thoroughly with soap before leaving the laboratory to ensure that you do not take any chemicals outside the laboratory. When working with radioisotopes such as ^{32}P, it is necessary to check your hands and shoes with a Geiger counter before leaving the laboratory.

The laboratory will be equipped with safety showers, eyewash stations, emergency exits, sharps containers, and fire extinguishers. Take the time to become familiar with the location of all of these safety components. All "sharps" (razor blades, Pasteur pipettes, broken glass, etc.) should be placed in the labeled "sharps" containers. Your laboratory supervisor will instruct you on the proper use and disposal of all hazardous reagents. If you do become injured or have any questions about your health risk during the course of the experiment, *immediately* notify the laboratory instructor. Most laboratory supervisors have had training in dealing with fires and exposure to different chemicals. Have fun with the experiments, be safe, and always leave a clean laboratory workbench for the beginning of the next laboratory session.

Units of Biochemistry

Biochemistry employs a decade system of units based on the metric system. Thus, biochemists use units such as the mole or the liter and various subdivisions that differ by three orders of magnitude (Table I-1). With knowledge of the molecular weight of a particular molecule and equation I-1, a given mass of a molecule can be converted to units of moles:

(I-1)

$$\text{Number of moles} = \frac{\text{number of grams of molecule}}{\text{molecular weight of molecule}}$$

As indicated in Table I-1, grams may be converted to milligrams and moles can be converted to millimoles simply by multiplying each of the appropriate values by 10^3. For example, 0.025 mol of a molecule is equal to 25 mmol:

$$0.025 \text{ mol} \times \frac{10^3 \text{ mmol}}{1 \text{ mol}} = 25 \text{ mmol}$$

Volume and mole values define the *concentration* terms of molar (M), millimolar (mM), and micromolar (μM) as shown in equation I-2:

Table I-1 Basic Units Used in Biochemistry

Mole Units	Liter Units
1 mole	1 liter
1 millimole (mmol) = 10^{-3} moles	1 milliliter (ml) = 10^{-3} liter
1 micromole (μmol) = 10^{-6} moles	1 microliter (μl) = 10^{-6} liter
1 nanomole (nmol) = 10^{-9} moles	1 nanoliter (nl) = 10^{-9} liter
1 picomole (pmol) = 10^{-12} moles	
Gram Units	**Equivalent Units**
1 gram	1 equivalent (Eq)
1 milligram (mg) = 10^{-3} g	1 milliequivalent (mEq) = 10^{-3} Eq
1 microgram (μg) = 10^{-6} g	1 microequivalent (μEq) = 10^{-6} Eq
1 nanogram (ng) = 10^{-9} g	
1 picogram (pg) = 10^{-12} g	
1 femtogram (fg) = 10^{-15} g	

(I-2)

$$\text{Concentration (molar)} = \frac{\text{number of moles}}{\text{volume (in liters)}}$$

$$\text{Concentration (millimolar)} = \frac{\text{number of millimoles}}{\text{volume (in liters)}} = \frac{\text{number of micromoles}}{\text{volume (in milliliters)}}$$

$$\text{Concentration (micromolar)} = \frac{\text{number of micromoles}}{\text{volume (in liters)}} = \frac{\text{number of nanomoles}}{\text{volume (in milliliters)}}$$

Similarly, volume and equivalent values define the *concentration* term of normality (N) commonly used in the expression for acid (H^+) or base (OH^-) strength, as indicated by equation I-3:

(I-3)

$$\text{Concentration (normal)} = \frac{\text{number of equivalents}}{\text{volume (in liters)}} = \frac{\text{number of milliequivalents}}{\text{volume (in milliliters)}}$$

Because these units involve basic metric principles, one can make use of the metric interconversions of mass (grams), fluid volumes (liters or milliliters), and spatial volumes (cubic centimeters, cc). Specifically, under most laboratory conditions, 1 ml of water or dilute aqueous solution weighs approximately 1 g and occupies 1 cc of volume (1 ml = 1.000027 cc).

These simple interrelationships of moles, weights, volumes, and so forth are often covered in introductory or freshman level college chemistry textbooks. Yet, practical experience reveals that these basic concepts are a major source of difficulty for many students in their initial exposure to experimental biochemistry. Therefore, we strongly suggest that students thoroughly review these concepts before conducting the experiments described in this textbook.

Analysis and Interpretation of Experimental Data

In nearly all of the experiments outlined in this textbook, you will be asked to collect, analyze, and interpret experimental data. Whether you are determining the concentration of a molecule in an unknown solution, the activity of an enzyme, the absorbance of a solution at a particular wavelength, or the activity of a particular isotope in a biological sample, the exercise will require you to perform a quantitative measurement and calculate a specific value. There are several questions that frequently arise during the analysis of experimental data: How

do you determine the level of precision of a set of measurements? How many data values or trials of an experiment must you perform before a measurement can be deemed precise? If you have a single value in a data set that is not in agreement with other members of the set, how do you determine whether it is statistically acceptable to ignore the aberrant value? In the subsections below, each of these issues is addressed.

Accuracy, Precision, and Bias of a Quantitative Measurement

When interpreting laboratory data, it is important to recognize that individual measurements, such as the concentration of a biological molecule observed in an assay, are never entirely accurate. For instance, the serum cholesterol measured by a medical laboratory from a blood sample is not the exact average serum cholesterol in the patient's blood at the time the sample was drawn. There are a number of reasons for this, the most obvious being that cholesterol may not have been quite uniformly mixed throughout the bloodstream. The patient's blood and the sample drawn from it are never totally homogeneous, the reagents used in the test are never totally pure or totally stable if repeatedly used, and the calibration of the autoanalyzer is never exactly correct or totally stable. Even such small deviations from the ideal execution of the assay may sometimes noticeably affect the results, and additional undetected errors in execution sometimes produce substantial errors. For these reasons, carrying out the same experiment more than once, or even repeatedly assaying the same sample, is bound to produce somewhat different numerical results each time.

Now, although the quantity being measured is a property of the particular sample under study, the degree of expected fluctuation from one measurement to another depends most fundamentally on the measurement process itself—that is, how the assay is conducted—rather than on the particular sample. Since, depending on the circumstances, the amount of fluctuation among attempts to measure the same quantity may be trivial or crucially important, we now consider briefly some basic concepts that help the biochemist deal with variability among measurements.

In performing an assay, the biochemist aims for *accuracy*. An assay method is accurate when the chance is high that its result will be quite close to the true value being measured. Since individual assay results invariably fluctuate, an accurate assay method must be (1) highly *precise* (equivalently, reproducible), having little variability when repeated, and (2) nearly *unbiased*, meaning that almost all of the time the average result from a large number of repeated assays of the same sample must be very close to correct. Conversely, an assay method can be poor because it is imprecise, biased, or both. For instance, a highly reproducible assay based on a very poorly calibrated instrument may yield almost the same, but grossly incorrect result, every time it is applied to a given sample. Another assay may be unbiased but also never close to correct, because its frequently large overestimates are balanced out by equally large and frequent underestimates.

The above concepts become more precise when expressed mathematically. Let the Greek letter μ represent the true characteristic of a sample that we are trying to measure, and suppose the n observations x_i, $i = 1, \ldots, n$, represent the results of entirely separate executions of an assay procedure. Then $(x_i - \mu)$ is the error of the ith assay. If we square these errors and take their arithmetic mean, we obtain the *mean squared error* $= (1/n)\Sigma(x_i - \mu)^2$ of the group of n repetitions. If we could repeat the assay an extremely large number of times, so that n is very large, the average result $\bar{x} = (1/n)\Sigma x_i$ would eventually stabilize at a limiting value $\bar{X}$, the "long-run" average value of the assay for the given sample. The mean squared error would similarly stabilize at a value, MSE, that can be used as an index of the assay's inherent accuracy. In principle, a perfect assay method has MSE = 0 for all samples, but no such assay exists. The bias of the assay is $(\bar{X} - \mu)$ and its square, $(\bar{X} - \mu)^2$, is a component of the MSE. The difference $\text{MSE} - \text{Bias}^2 = \text{MSE} - (\bar{X} - \mu)^2 = \sigma^2$ is a measure of inherent variability of the assay, known as its *variance*. While we can never determine the variance of an assay exactly, because that would require performing the assay an impossibly large number of times, we can estimate it by

$$s^2 = \frac{1}{n-1} \sum (x_i - \bar{x})^2$$