Experiments in Biochemical Research Techniques

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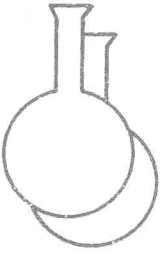
EXPERIMENTS

IN

BIOCHEMICAL RESEARCH TECHNIQUES



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PREFACE

This book contains a selection of experiments that are intended to illustrate some of the major research techniques of modern biochemistry. For the past few years, these experiments have constituted the elective and required laboratory material for two consecutive one-semester courses attended by first-year graduate students in biochemistry and related fields at the University of California (Berkeley). For these courses, we have selected a limited number of experiments of moderate complexity in order to teach the techniques within a reasonable time. Also, we have tried to develop a "research attitude" in our students by presenting the experiments in such a way that the students must acquire some of the details from their own reasoning and ingenuity or from references to the literature.

We must justify the need for a course in advanced biochemical techniques, for it may reasonably be asked whether graduate students could not spend their time more profitably in other pursuits, for example, in actual research. It is true that the student can acquire the techniques presented in this book from another research worker when the need arises; or he may even work out the methods for himself if necessary. However, these alternative ways of learning are themselves time-consuming and inefficient. What is more important, one tends to apply to a research problem those methods with which one is familiar and hesitates to take the time to learn new methods; hence, quite useful techniques may never be applied because of an "inertia of ignorance." Therefore, we feel

that it is worth our students' time to become acquainted, through a laboratory course, with a variety of the techniques that they will need most in their research. This manual was written to provide the necessary experiments, since no published compilation was available.

The question of which techniques are the most important also must be considered. It is far too much to expect that everyone should hold the same ideas on this subject. The most we can hope is that our selection will satisfy a few people, will be acceptable in part to many others, and therefore will provide a selection of useful experiments. A number of alternative experiments have been provided to permit a wider choice. We have omitted experiments of the kind given in basic biochemistry courses because they should have been performed earlier by the students for whom this manual is intended. A number of manuals that contain these basic experiments are available. Certain highly specialized techniques that require elaborate equipment (such as ultracentrifugation and boundary electrophoresis), microbiological and hormone assays, and nutritional experiments have been omitted. Experiments based extensively on organic synthesis or degradation were not included. To recapitulate, selection was necessary in order to keep the amount of work expected of the students within reasonable limits of time and effort, and our choice has been based primarily upon our interests and experience. We shall be most grateful for suggestions regarding either the individual experiments or the selection of experiments in this manual.

We should like to express our gratitude to Dr. Willis H. Reisen who assembled certain of the experiments in Sections I and III of this book. We also are indebted to Dr. A. K. Balls of Purdue University, Dr. A. L. Marr of the University of California at Davis, Dr. L. K. Noda and Dr. F. W. Strong of the University of Wisconsin, and Dr. Harland Wood of Western Reserve University for providing us with experiments used in their courses. Our greatest debt is to our students and our teaching assistants who tested these experiments and who suggested many improvements.

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May 1, 1957

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GENERAL INTRODUCTION

The experiments in this manual are intended for advanced students and many of them are designed as problems in research, not as routine laboratory exercises. Consequently, the procedures described are in some cases little more than guideposts to you, the researcher. You will be expected to give some thought to modifications of experimental procedures, and to supplement the text instructions by reading the original literature for more details.

Two rules for your safety are to be stressed.

a. No student is to work in the laboratory when he is alone.

b. Students are not to smoke nor have flames near apparatus unless sure the contents are non-flammable.

Every experiment must be clearly described in your notebook. The essential characteristic of a good laboratory notebook is that it be sufficiently detailed and neat so that someone else (or you yourself) could repeat the experiment and obtain comparable results. The following material should be included in your notebook.

1. Date of the experiment.
2. Title: a quick reference to the subject of the experiment.
3. Purpose: a clear statement of the questions the experiment is designed to answer.
4. References: a citation of essential literature.
5. Procedure: a description clear enough for duplication of the experiment at a later date. You need not copy material from other sources un-

less major changes have been made, but a reference should be provided.

6. Results: a direct record of data (which subsequently may be reorganized in the form of tables or charts). If it is necessary to use results obtained by other students, the source of the data should be acknowledged.

7. Discussion and conclusions: a brief summary of your discoveries and their relation to results obtained by others. Such a summary is very useful later, when you wish to recall quickly what the experiment showed.

You will find that each experiment in this manual is arranged under certain headings (Objectives, Principles, etc.). The Objectives of each experiment are stated first. Before commencing your laboratory work you should understand the purpose of a particular experiment and what you are expected to learn from it.

The Principles, given next in some experiments, are brief summaries of background material and other information useful for the experiment. The major references given in this section are especially important and you are expected to read them before beginning the actual experimental work.

The list of Principal Equipment and Supplies contains only the less common pieces of apparatus, solutions, and chemicals you will need for the experiment. It is your responsibility, however, to see that everything required is ready when you need it. Be certain, for example, that such things as sterile media, cold solutions, bacterial cultures, and warm-water baths are prepared ahead of time.

The Procedure should be read completely before the experimental work is commenced. The point to be stressed is that you should know what you are going to do and why, before you come to the laboratory. The following general suggestions for working in the laboratory are offered at this point.

a. Perform odd jobs such as cleaning equipment or calculating results in the intervals that occur during an experiment between major operations. This sort of planned procedure is a key to efficient laboratory work; you should never find yourself with nothing to do.

b. Use clean equipment and keep your desk neat while you work.

c. You will not become so tired while you work if you sit down whenever possible.

d. When you have a question, try to answer it for yourself before you see the instructor. Ask yourself what experiments you would have to do to obtain an answer; you may find that you already have the information required to answer the question.

e. Try to develop some original ideas about the experimental procedures, and try to plan more interesting experiments. Check with the instructor before attempting any major change, however, to be sure that it is feasible and that equipment is available. There are often good reasons for the specific details in the given procedures.

The section on Treatment of Data is deliberately varied from one experiment to another to help you to develop some skill in reporting scientific work. Reports are oral or written, formal or informal. Formal, written reports are usually the most difficult to prepare but books are available to help you in this task (1, 2). Brief and clear directions on how to revise your report for greater clarity and better grammar also have been published (3).

The Questions section of each experiment is designed to bring to your attention concepts and procedures that were not stressed in the text of the experiment.

The References listed at the end of each experiment have been kept to a minimum number. The major references, numbered in underlined type, are articles which will help you to acquire a clear understanding of the important points of each experiment. At least the portions of these references that are applicable to the experiments should be read. Supplementary references generally contain information of a more specialized nature.

It is most important for a scientist to read the literature. In actual research, much time in the laboratory can be saved by reading pertinent articles both before starting a new problem and again after work has begun. During these experiments, too, you should read the essential material in the literature. A second reason for reading original articles and reviews is to broaden your background and acquire new concepts. The importance of this reading cannot be overestimated. You should develop a habit of skimming through titles and summaries of the current articles in several Journals. It is impossible to read even a fraction of the total that appears, and you should read an entire article only when it becomes essential for your research.

Biochemical literature appears in different forms, and serves different purposes.

1. Biochemistry texts are useful to gain a preliminary, very general view of a topic. The treatment of specific problems is too brief for use in research and the texts may be out of date by five years or more in regard to specific points.

2. Specialized reference books, such as The Enzymes (4), will provide summaries of basic knowledge and are especially useful for references to earlier papers of importance.

3. Once you have gained a general idea of your topic, some recent specialized review can be extremely helpful. Annual Reviews (of Biochemistry, Microbiology, Physiology, etc.), Advances (in Enzymology, Carbohydrate Chemistry, etc.), or similar review series are examples. Start with the most recent volume and work backwards; note references to pertinent original papers and reviews.

4. Abstract Journals (Chemical Abstracts, Biological Abstracts, etc.) are useful for locating all published work except that appearing in the past year. However, it is often easier to find important papers in reviews than in abstracts.

5. The current Journals must be searched for articles published within the past year. The references listed in review articles should give you an indication of the Journals that are likely to carry pertinent articles. In reading articles, start with the recent ones, for they will provide references to earlier work.

6. Abstracts of recent scientific meetings (Federation Proceedings, American Chemical Society Meetings, etc.) contain brief descriptions of very recent, unpublished work, and these indicate what work is likely to be going on at present.

Some references may be made to books dealing with the way biochemi-

cal research is actually performed. A most worthwhile book by Beveridge (5) points out, among other things, that keen observation in the laboratory and a questioning attitude toward unexplained results lead to many important discoveries, and also that an initial step in research often arises from intuition and inspiration of an artistic sort. Reason later provides a check. A most interesting book on the scientific life of Pasteur (6) is valuable reading for students looking toward a career in science.

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section I

PHYSICAL CHEMICAL METHODS FOR SEPARATION AND IDENTIFICATION OF BIOLOGICALLY IMPORTANT COMPOUNDS

Frequently an investigator in the field of biochemistry is faced with the problem of separation and identification of compounds in a mixture, the components of which are structurally similar and, therefore, possess very similar physical and chemical properties (mixtures of nucleotides, of fatty acids, of proteins or their degradation products, etc.). Often only a partial separation of structurally similar compounds is effected by a single performance of a given separation process, such as a single extraction or a single adsorption on charcoal. Where separation does depend upon subtle differences in physical or chemical properties, a repetition of the basic separation process is required in order to approach complete separation. This may take the form of a cascade-like repetition of the unit process; thus countercurrent distribution may be viewed as a systematic, repetitive process of distribution between two liquid phases. Or, the repetition may take the form of a continuous, non-differentiated process in which unit steps are no longer distinguishable; thus partition column chromatography may be viewed as a process of continuous distribution and redistribution of solutes between two phases throughout the column. Because of their extreme effectiveness, many of the methods of separation and identification described in this section are of the continuous, non-differentiated type (Table I).

This section will be limited to certain methods of wide applicability for which apparatus is likely to be available or readily procurable. The section does not include such highly useful methods for the study of large

TABLE I. TECHNIQUES FOR SEPARATION BY PHASE DISTRIBUTION

Type of Separation Process	Phase Pairs Involved		
	Vapor-Liquid	Liquid-Liquid	Liquid-Solid
Batchwise	Simple distillation	Extraction	Decolorization by adsorption
Cascade	Bubble-cap distillation	Countercurrent distribution	Systematic, repeated adsorption
Continuous, non-differentiated	Packed column distillation	Liquid-liquid partition chromatography	Adsorption chromatography
	Gas-liquid partition chromatography	Paper chromatography	Ion exchange chromatography

molecules as the ultracentrifuge, boundary electrophoresis, light scattering, etc., because of the elaborate equipment required. Also, such rather simple separation procedures as the commonly used isolation procedures for proteins, fats, nucleic acids, etc., are not considered in this section.

A. Distillation at Low Pressures

Distillation is the oldest of all techniques described in this section and is, in fact, one of the oldest of all chemical processes. It may be employed for purification of a compound or for proximate analysis of a mixture. Amounts of material from about two grams to several kilograms may be handled in suitably designed laboratory apparatus. The components of the typical distillation system for pressures down to about 1 mm of Hg are shown in Figure 1. Descriptions of the multitude of types, capacities, and pressure ranges of the various components of the distillation system are set forth in refs. (1) and (2). In assembly of such a system, one should first establish the desired capacity and operating pressure ranges, then consult the above references for the best combination of components for the given system.

Although low-pressure distillation encompasses all distillations at less than atmospheric pressure, most fractional distillations at low pressure are conducted in the range of 1 – 20 mm of Hg. One advantage of low pressure for distillation of biologically important compounds is the decreased thermal decomposition at the lower distillation temperature, and a possible second advantage is a change in relative volatility of components; for example, ethanol and water form constant boiling (azeotrophic) mixtures at atmospheric pressure but do not do so at pressures below 70 mm of Hg and can be readily separated at low pressures.

Certain biological materials such as the oil-soluble vitamins require

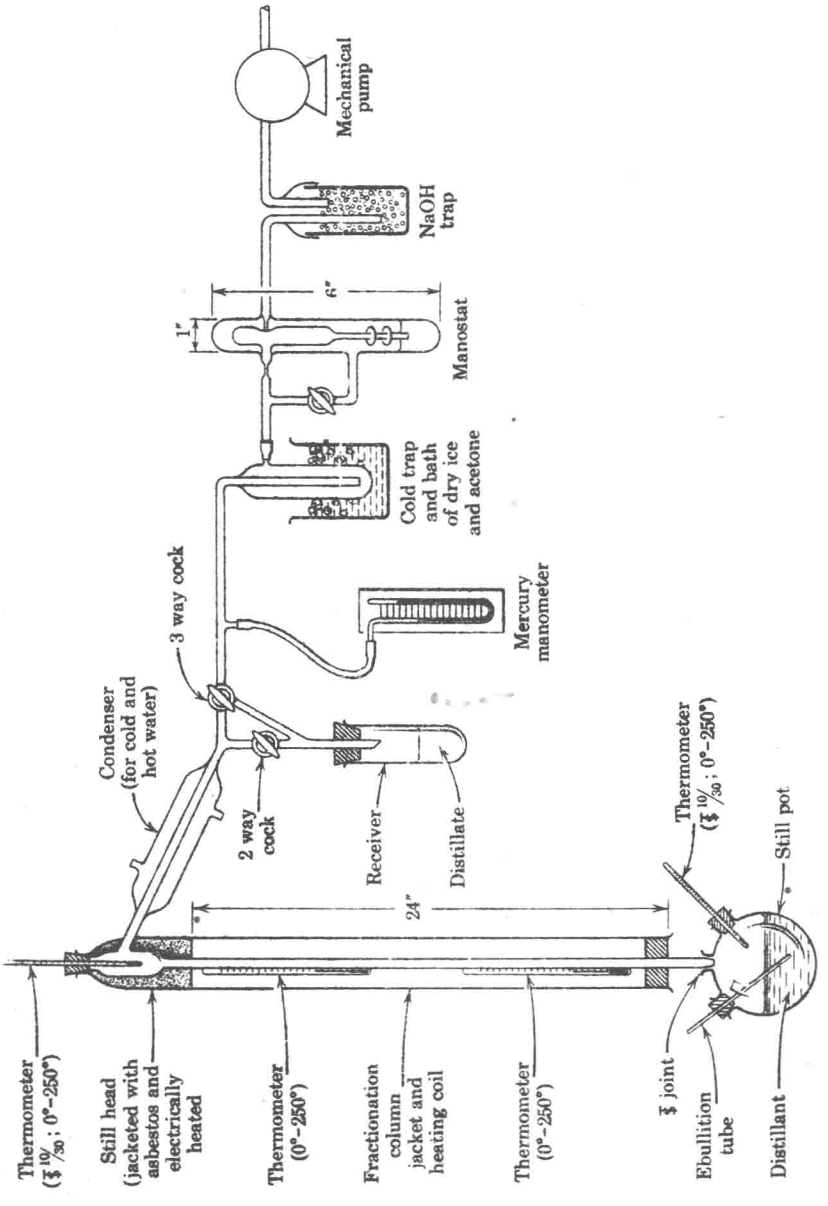

Figure 1. Apparatus for distillation at low pressures.

very high temperatures for distillation, even at 1 mm of Hg. For these compounds and for certain other compounds of molecular weights as high as 1200, molecular distillation may be used. Molecular distillation is a process of molecular diffusion from a hot layer of solid or liquid distilland to a cooled condenser. The condenser is placed at a distance from the surface of the distilland which is slightly less than the mean free path in the vapor phase of the molecules to be distilled. Molecular distillation is thus a distinctly different process from fractional distillation at low pressures; it utilizes much the same type of vacuum system, although components must be chosen for operation at pressures below 10μ (1μ = 0.001 mm of Hg).

References*

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2. Rose, A., and Rose, E. 1951. Theory of Distillation. In Technique of Organic Chemistry, IV, 1–174. A. Weissberger, editor. Interscience Publishers. New York.

*Major references are designated throughout this book by underlined reference numbers.

EXPERIMENT 1. LOW PRESSURE DISTILLATION OF METHYL ESTERS OF FATTY ACIDS (4 periods)

OBJECTIVE

This experiment will provide experience in the operations of vacuum distillation and an appreciation of the characteristics of various types of apparatus.

PRINCIPAL EQUIPMENT AND SUPPLIES

Twenty-four-inch Podbielniak column with partial reflux head (ref. [1], p. 237), and/or

Thirty-inch Vigreux column with variable take-off head (ref. [1], p. 245), or other columns of comparable efficiencies at low pressures

Mechanical pump

Auxiliary distillation equipment shown in Figure 1

Abbé refractometer and circulating water bath at 45°

Steam bath

Sample of coconut oil of unknown composition

Absolute methanol

Saturated sodium chloride

0.5 N alcoholic KOH

0.5 N HCl

Bromphenol blue (0.04%)

Phenolphthalein in alcohol (1%)

Methyl orange (0.04%)

PROCEDURE

You should be familiar with the theory of fractional distillation and the operation of the equipment before you attempt this experiment. It is recommended that the references listed at the end of this experiment be consulted; in particular, ref. (1).

1. *Formation of Methyl Esters of the Fatty Acids* (2)

To 100 gm of coconut oil in a 500 ml round-bottom flask are added 250 ml of absolute methyl alcohol and 5 ml of concentrated H_2SO_4. After the mixture has been refluxed on the steam bath for 20–24 hr an equal volume of saturated sodium chloride solution is added, and the aqueous phase of the mixture is neutralized to methyl orange with powdered sodium carbonate. The layer of mixed methyl esters is separated, washed twice with water, and dried by heating to 110° in a beaker for 30 min.

2. *Fractional Distillation of the Methyl Esters*

The oily liquid is transferred to a three-neck, 250 ml, round-bottom flask with standard taper fittings. The center neck joins the distillation