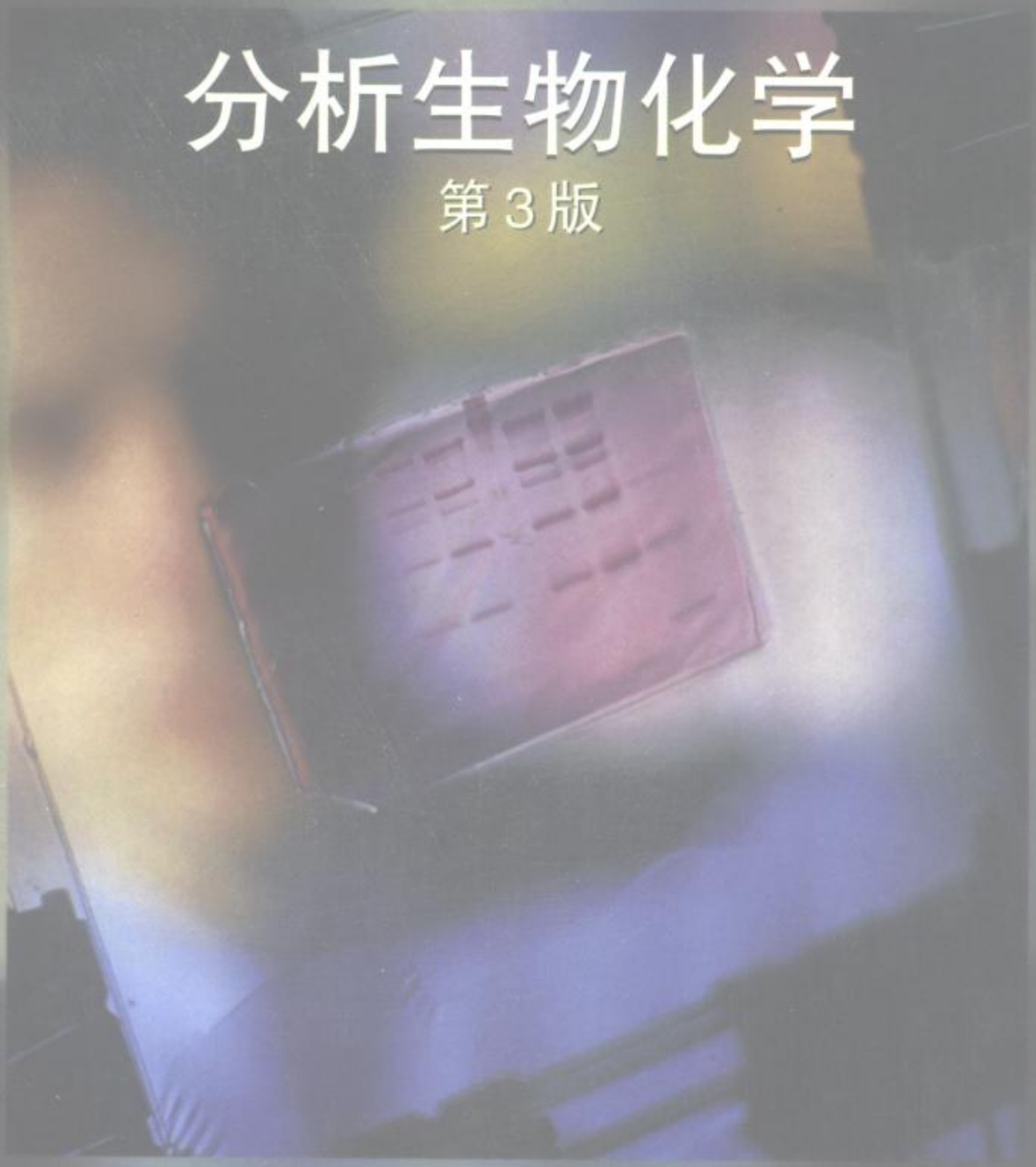
THIRD EDITION

ANALYTICAL BIOCHEMISTRY

分析生物化学

第3版



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Analytical Biochemistry

Third Edition

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Analytical Biochemistry

Third Edition

Preface

TO THE THIRD EDITION

The technology associated with biochemical analysis is rapidly changing and new laboratory instruments are constantly being introduced. However, with a few exceptions, the innovations are not based on new principles of analysis, but offer analytical benefits often through a 'mix and match' approach. For example, modern HPLC instruments may use columns that combine various features to effect separation and offer a range of detector options; the boundaries between electrophoresis and chromatography become blurred in such techniques as capillary chromatography; and mass spectrometry, previously only associated with GLC, is now linked to a much wider variety of chromatographic techniques. These 'state of the art' instruments are normally microprocessor controlled, offer some degree of automation and are attractively designed for ease of use.

Against this background it is perhaps tempting for analysts to underestimate the importance of understanding the principles of the techniques they are using. Unless this is the case they will be unlikely to be able to select, optimize and develop new methods, troubleshoot existing ones and be confident in the quality of their results. With increasing importance being attributed to quality assurance and laboratory accreditation, in addition to the fact that employers require their staff to work efficiently, an appreciation of fundamental principles of analysis is vital. We therefore make no apologies for again concentrating on these in this third edition.

We have deleted some sections that contained detailed accounts of techniques that are rarely encountered in modern laboratories, while retaining reference to the important classical methods that do provide the basis of current methodology. New material has been added to bring some topics up to date and these include increased coverage of laboratory quality, safety and accreditation, use of kits, mass spectrometry, and capillary electrophoresis. Many other changes have been made, not least of which is a completely new layout of the typescript with boxed areas for emphasis. We hope this will aid understanding and make the book more 'user friendly'. Two types of self test question are also included, which are designed to be simple indicators of an understanding of the basic concepts of the section and not a comprehensive test of knowledge of the topics. We have decided not to include photographs of particular instruments, as they are often not particularly informative and the designs change so rapidly. We would like to thank Dr Susan Laird and Dr Robert Smith for revising their chapters on nucleic acids and immunological

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methods respectively. We are also indebted to the many colleagues who have shared their knowledge and expertise with us over the years and whose advice has been invaluable.

Analytical biochemistry is an extensive subject and both the actual content and the balance of coverage in such a book as this is open to debate. With this in mind, our aim in each edition has been to give a clear account of the principles of the subject that will aid the understanding of a wide range of scientists who are either studying for a qualification or who are working in a laboratory, or perhaps both. The reading lists at the end of each chapter suggest additional texts for readers who require more details of specific topics.

David J. Holme
Hazel Peck

April 1997

Preface

TO THE SECOND EDITION

Since the publication of the first edition in 1983, several specialist books which cover a range of specific techniques in detail have been published. However, the ability to select an appropriate technique for a particular analytical problem still remains fundamental and the first edition of this book evidently proved useful in this respect. Thus the principal objective for this second edition remains unchanged.

Much of the information has been updated for the second edition to reflect substantial changes in the subject. The edition of a chapter on nucleic acids was considered essential and complements the original chapters on the chemical nature and methods of analysis of other important biological molecules. We are indebted to Dr Susan Laird for compiling this chapter and also to Mr Robert Smith for the major update on immunoassays in the immunological methods chapter.

We have maintained the same balance of information in the new chapter and therefore details of specific applications of techniques are not discussed, for example, DNA fingerprinting. Where appropriate, we have included titles of books which have an emphasis on applications in the further reading list at the end of each chapter. These lists are not intended to be fully comprehensive, nor are the chapters referenced as we consider this to be inappropriate for the level of potential readership.

We have received many pleasing reports of the usefulness of the first edition in a range of analytical laboratories, in areas such as pharmaceuticals, biotechnology, agrochemicals, clinical biochemistry, molecular biology, etc. Our own experience and comments from colleagues in other universities have reinforced our initial purpose of writing a book for students on a range of courses that include the analytical aspects of biochemistry. We are therefore delighted that this softback edition is now available which will encourage wider access for student use.

Hazel Peck
David Holme
Sheffield Hallam University

July 1992

Preface

TO THE FIRST EDITION

The initial stimulus for writing this book arose out of difficulties experienced in recommending a single suitable textbook for students on courses in which the analytical aspects of biochemistry were a major component. Although there are many books on analytical chemistry in general and clinical chemistry in particular, many omit the biochemical aspects of analysis such as enzymology and immunology while others do not cover the basic science of the subject. The objective was to bring together in one book those topics which we consider to be essential to the subject of analytical biochemistry.

In the introductory section to each chapter, there is a brief explanation of the scientific basis of the topic and this is followed by a discussion of the analytical methods which are relevant. While it is not intended that it should be a book of 'recipes', technical details for many of the methods described are given. This will help those readers with no practical experience to appreciate the steps involved in the analysis while at the same time giving sufficient detail for the method to be developed in practice. It is intended that the book will provide enough information to enable a student to select a technique or series of techniques which would be appropriate for a particular analytical problem and to be able to develop a valid and reliable analytical method.

The topics covered in this book fall into three main groups. Analytical techniques such as spectroscopy, chromatography, etc. are particularly important in analytical biochemistry as well as in analytical chemistry generally. The principles of each technique are explained and the scope and applications are discussed. There are chapters on enzymes, antibodies and radioisotopes, substances which it may be necessary to detect and measure but which also can be very useful in a variety of analytical methods. Here again, the basic theory is explained before discussing their applications in analytical biochemistry. Finally, there are four chapters which explain the chemical nature and methods of analysis of the major groups of biologically important compounds, namely, carbohydrates, amino acids, proteins and lipids. While it is appreciated that the range of compounds in this final section could be considerably extended it has been deliberately restricted to those groups which we consider to be of particular biochemical importance.

At the end of each chapter, several books are listed for further reading on the subject but it is suggested that the following books would be suitable for further reading on the topic of biochemistry of amino acids, carbohydrates, proteins and lipids.

J.W. Suttie, *Introduction to biochemistry*. Holt, Rinehart and Winston, New York, USA.
H.R. Mahler and E.H. Cordes, *Biological chemistry*. Harper and Row, New York, USA.
A. White, P. Handler and E.L. Smith, *Principles of biochemistry*. McGraw-Hill Book Co., New York, USA.

We would like to thank Dr Rodney Pollitt for reading the draft text and for his invaluable comments. In addition, we would like to thank those colleagues who have helped in various ways and Mrs P. Holme for typing the manuscript.

David J. Holme
Hazel Peck
Sheffield City Polytechnic

February 1982

About the boxes and self test questions

The book contains margin notes and two types of boxes, which are designed to enable the reader to identify certain types of information easily.

The margin boxes highlight important points in the text with a short statement or definition to give some background to the topic or they refer to other sections in the book which give additional information on the topic.

The procedure boxes give technical details of some procedure either to illustrate a technique or to provide technical details for readers who wish to use it.

The self test questions are at the end of most sections in a box. The four questions are designed to test the reader's understanding of the basic principles of the topic without going into the details of the subject. There are two types of questions:

1. Multiple choice question – any number or none of the alternatives may be correct.
2. Relationship analysis – consists of two statements joined by the word BECAUSE. Each statement should be considered separately and identified as being either TRUE or FALSE. If both statements are true, then the whole sentence should be considered to decide whether, overall, it is correct (YES) or not (NO), i.e. whether the second statement provides a correct explanation for the first statement. It should be appreciated that one short statement will not provide a complete explanation but the overall sentence can still be true.

Contents

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1 General principles of analytical biochemistry

Key topics

- The selection of a valid method of analysis
- The quality of data
- The production of results

Analytical biochemistry involves the use of laboratory methods to determine the composition of biological samples and it has applications in many widely differing areas of biological science. The information gained from an analysis is usually presented as a laboratory report, which may simply say what substances are present (a qualitative report) or may specify the precise amount of a substance in the sample (a quantitative report).

➤ A qualitative method enables only the presence of the substance to be detected.

A **qualitative report** will often indicate whether a particular substance or group of substances is present without commenting on the complete composition of the sample. In many cases the report will also specify the individual members of that group of substances. It might, for instance, name only the different carbohydrates present although the sample contained other substances, e.g. lipids and proteins. It is possible, when using some qualitative methods, to compare the amount of substance in the sample with the amount in a reference sample and to report the presence of either increased or decreased quantities. Such a report is said to be **semi-quantitative**. Chromatographic and electrophoretic methods often give results which can be interpreted in this way.

➤ A quantitative method enables the amount of substance present in a sample to be determined.

A **quantitative report** will state the amount of a particular substance present in the sample and it is important that the units of measurement are meaningful and appropriate in order to prevent subsequent misunderstandings. When reporting quantitative results it is desirable to indicate their reliability, a feature which can often be assessed statistically. In practice it may not be necessary to present this information with each report but it should be readily available for reference.

1.1 The selection of a valid method of analysis

In order to be able to choose a suitable analytical method it is essential to know something about the chemical and physical properties of the test substance (Table 1.1). Because the relationship between the property and the amount of substance is not always a simple one, some methods are only suitable for the detection of the substance (qualitative) while others may be quantitative. For any method it is important to appreciate the nature of the relationship between

Table 1.1 Physical basis of analytical methods

Physical properties that can be measured with some degree of precision	**Examples of properties used in the quantitation of**		
	Protein	Lead	Oxygen
Extensive			
Mass	+	+	
Volume			+
Mechanical			
Specific gravity	+		
Viscosity	+		
Surface tension	+		
Spectral			
Absorption	+	+	
Emission			
Fluorescence			
Turbidity	+		
Rotation			
Electrical			
Conductivity			
Current/voltage			+
Half-cell potential			+
Nuclear			
Radioactivity			

Proteins are the major components by bulk in many biological samples and hence the weighing of a dried sample should give an estimate of the amount of protein present. Similarly, solutions that contain protein show values for specific gravity and surface tension which are in some way related to protein content. Measurements of the turbidity resulting from the precipitation of protein and the absorption of radiation at specific wavelengths have all been used quantitatively.

The lead content of biological samples is usually very small, rendering gravimetric methods impracticable, and methods have often relied upon the formation of coloured complexes with a variety of dyes. More recently, the development of absorption spectroscopy using vaporized samples has provided a sensitive quantitative method.

Oxygen measurements using specific electrodes offer a level of sensitivity which is unobtainable using volumetric gas analysis.

the measurement obtained and the amount of substance in the sample.
Most analytical methods involve several preparative steps before the final measurement can be made and it is possible to produce a flow diagram representing a generalized method of analysis (Table 1.2). Not all the steps may be necessary in any particular method and it may be possible to combine two or more by careful choice of instrumentation. It is important when selecting a particular method to consider not only its analytical validity but also the cost of the analysis in terms of the instrumentation and reagents required and the time taken.

Table 1.2 Generalized method of analysis

The major manipulative steps in a generalized method of analysis
Purification of the test substance
↓
Development of a physical characteristic by the formation of a derivative
↓
Detection of an inherent or induced physical characteristic
↓
Signal amplification
↓
Signal measurement
↓
Computation
↓
Presentation of result

1.1.1 Instrumental methods

The most convenient methods are those that permit simultaneous identification and quantitation of the test substance. Unfortunately these are relatively few in number but probably the best examples are in the area of atomic emission and absorption spectroscopy, where the wavelength of the radiation may be used to identify the element and the intensity of the radiation used for its quantitation.

➤ Atomic spectroscopy – see Sections 2.2 and 2.5.

If a compound does not show an easily detectable characteristic it may be possible to modify it chemically to produce a compound which can be measured more easily. In the early part of this century, this approach to analysis

led to the development of many complex reagents designed to react specifically with particular test substances. Generally these reagents resulted in the formation of a colour which could be measured using visual comparators. Most of these reagents have been superseded by improved instrumental methods but some very reliable ones still remain in use. They were often named after the workers associated with their development, e.g. Folin and Ciocalteu's reagent, originally described in 1920 for the detection of phenolic compounds.

➤ Interference occurs when other substances as well as the test substance are detected by the method.

Interference occurs when other substances, as well as the test compound, are also detected, resulting in erroneously increased values. Occasionally interference effects can result in suppression of the test reaction. For any method it is important to be aware of substances that may cause interference and to know if any are likely to be present in the sample.

If interference is a major problem the sample must be partially purified before analysis. This breaks the analysis into preparatory and quantitative stages. In order to reduce the technical difficulties resulting from such two-stage methods much work has gone into the development of analytical techniques such as gas and liquid chromatography in which separation and quantitation are effected sequentially.

➤ Chromatography – see Section 3.2.

1.1.2 Physiological methods

While it may be possible to devise quantitative methods of analysis for many biochemical compounds, the only practical method of measurement for others is through their physiological effects. A **bioassay** involves the measurement of a response of an organism or a target organ to the test compound and may be conducted *in vivo* using live animals or *in vitro* using isolated organ or tissue preparations. Many bioassays are quantitative but those that give only a positive or negative result are said to be quantal in nature.

➤ Bioassays measure the response of living cells to external factors.

A satisfactory bioassay demands that the response of the animal to the substance can be measured in some fairly precise manner but it must be remembered that different animals respond in different ways to the same stimulus. Bioassays must therefore be designed to take account of such variations and replicate measurements using different animals must be made. In all assays it is important that the external factors that may influence the response are standardized as much as possible. The age and weight of an animal may affect its response as may also the environmental conditions, route of injection and many other factors.

In the absence of absolute chemical identification it is often necessary to establish that different samples contain the same physiologically active substance. This may be achieved by comparing the dose–response relationship for both samples. This involves measuring the response to varying amounts of each sample and demonstrating that the slope of the resulting relationship is the same in both cases. In such graphical or statistical methods it may be necessary to use the logarithm of the amount in order to produce a straight line rather than a curve. It is often necessary to use such a technique to confirm the validity of using synthetic or purified preparations as standards in quantitative assays.

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