
The Tools of Biochemistry

Terrance G. Cooper

The Tools of Biochemistry

Terrance G. Cooper

University of Pittsburgh

A Wiley-Interscience Publication

JOHN WILEY & SONS, New York / London / Sydney / Toronto

Copyright © 1977 by John Wiley & Sons, Inc.

All rights reserved. Published simultaneously in Canada.

No part of this book may be reproduced by any means,
nor transmitted, nor translated into a machine language
without the written permission of the publishers.

Library of Congress Cataloging in Publication Data:

Cooper, Terrance G. 1942-
The tools of biochemistry.

"A Wiley-Interscience publication."

Includes bibliographical references and index.

1. Biological chemistry—Technique. I. Title.

QP519.7.C66 574.1'92'028 76-30910

ISBN 0-471-17116-6

Printed in the United States of America

10 9 8 7 6 5 4 3 2 1

Preface

An essential condition for fruitful research is to have satisfactory techniques at one's disposal. Progress in biological sciences has largely depended on parallel advances in the technology of measuring and observing devices. Unfortunately, a lack of understanding of the tools used is commonly a weakness of many investigations. Careful evaluation is needed, not only of experimental data but also of the methods by which they were obtained.

The purpose of this work is to increase the number and quality of biochemical techniques available to the reader. Often students, during their undergraduate and graduate studies, become acquainted with only the techniques used in their immediate environments. They may find it difficult to apply entirely new approaches and techniques to a problem because they lack a general source of information that presents the potential and limitations of a particular method as well as an opportunity to use it under well characterized conditions. It is to this difficulty that I address this book. Although the text forms the basis of a senior level undergraduate course, it is intended also to serve as a ready source of useful information for graduate students and experienced investigators. Its purpose is to enlarge the sphere of the reader's experience, and its success will be measured by the degree to which this occurs.

The format used is to first present the theoretical basis and limitations of each technique. This section provides an understanding of the reasons for procedures that are subsequently performed but is not designed to be an absolutely comprehensive treatise; an experimental section follows in which a number of experiments using the method are described in detail. The particular experiments were selected because they are straightforward to execute and provide clear examples of data that can be obtained when the method is used properly. To permit application of this material to the greatest range of teaching and research requirements, each chapter is a self-contained unit. It is therefore possible to select those methods that are appropriate to the reader's needs and resources.

I wish to express my appreciation to Professor David Krogmann for providing the early stimulus and enthusiasm needed to undertake this work. Without his help it would still be just an idea. Many of my students and colleagues have read these chapters and offered advice for their

improvement. This and the many hours spent by my wife Carol reading and rereading the text have avoided countless errors, confusing statements, and insults to the English language. I did not, however, follow their good advice on all occasions and am solely responsible for any problems that still remain. For technical preparation of the manuscript I am indebted to Mrs. Sandra Wight. The many photographs generously provided by independent investigators and by manufacturers of scientific equipment and supplies enhance this work and are greatly appreciated. To the staff at John Wiley and Sons I am grateful for unlimited patience and assistance during development and publication of this work.

T. G. COOPER

Pittsburgh, Pennsylvania

January 1977

The Tools of Biochemistry

Contents

| | |
|--|-----------|
| Chapter 1. Potentiometric Techniques | 1 |
| pH Calculations | 2 |
| pH Measurement Using Organic Indicator | |
| Molecules | 7 |
| Potentiometric Measurement of pH | 10 |
| Reference Electrode | 10 |
| Glass Electrode | 12 |
| Electrometer | 16 |
| Buffers | 19 |
| Ion Specific Electrodes | 22 |
| Experimental | 26 |
| Preparation of a new or Unused | |
| Combination Electrode | 26 |
| Titration of an Amino Acid | 28 |
| Titration of an Amino Acid in the | |
| Presence of Formaldehyde | 29 |
| Calibration of the Clark Electrode | 30 |
| Oxygen Uptake by <i>Saccharomyces cerevisiae</i> | 33 |
| Oxygen Uptake by Castor Bean | |
| Mitochondria | 33 |
| References | 35 |
| Chapter 2. Spectrophotometry | 36 |
| Spectrophotometer | 42 |
| Light Source | 42 |
| Monochromator | 44 |
| Sample Chamber | 49 |
| Detector | 50 |
| Experimental | 51 |
| Biuret Protein Determination | 51 |
| Lowry Protein Determination | 53 |
| Determination of Inorganic Phosphate | 55 |

| | |
|---|-----------|
| Determination of Nucleic Acids by the Orcinol Reaction | 56 |
| Park and Johnson Method for a Reducing Sugar | 57 |
| Determination of the pK_a for Bromophenol Blue | 59 |
| Spectral Characteristics of Biologically Significant Molecules | 62 |
| References | 63 |
| Chapter 3. Radiochemistry | 65 |
| Measurement of β Radiation | 68 |
| Scintillation Spectrometry | 70 |
| Use of a Scintillation Spectrometer | 84 |
| Counting Efficiency | 87 |
| Simultaneous Counting of Multiple Isotopes | 96 |
| Sample Preparation for Scintillation Counting | 99 |
| Determination of Radioactive Carbon Dioxide | 102 |
| Gas Flow or Geiger Counting | 103 |
| Statistics of Counting | 108 |
| Labeling Procedures | 113 |
| Experimental | 121 |
| Preparation of Aqueous and Organic Scintillation Fluid | 121 |
| Determination of the Scintillation Counter Balance Point | 121 |
| Determination of a β Spectrum | 122 |
| Effect of Gain on a β Spectrum | 122 |
| Alternative Method of Determining the Balance Point of an Isotope | 122 |
| Effect of a Quenching Agent on a β Spectrum | 124 |
| Counting Quenched Samples Using the Channels Ratio Technique | 124 |
| Radioactivity Determinations of Multiply Labeled Samples Using the External Standard Channels Ratio Technique | 126 |
| Alternative Method of Determining the cpm of ^{14}C and ^3H in Multiply Labeled Samples | 127 |
| Determining the Half-Life of ^{32}P | 129 |

| | |
|---|------------|
| Determination of the Plateau Value on a Gas Flow Counter | 129 |
| Determination of the Instrument Dead Time | 131 |
| Incorporation of ^3H -Leucine into <i>E. coli</i> Proteins | 132 |
| References | 134 |
| Chapter 4. Ion Exchange | 136 |
| The Exchanger | 138 |
| Preparation of the Exchange Medium | 144 |
| Chromatography | 147 |
| The Column | 147 |
| The Gradient | 151 |
| Column Elution | 155 |
| Sample Size | 155 |
| Ion Exchange Techniques for the Assay of Enzymes | 155 |
| Experimental | 157 |
| Separation of Organic Acids on Dowex Resins | 157 |
| Separation of Amino Acids from Organic Acids on Dowex Resins | 162 |
| Separation of Nucleotides on Dowex Formate Columns | 165 |
| Assay of Acid Phosphatase Using Mini-Ion Exchange Columns | 165 |
| References | 167 |
| Chapter 5. Gel Permeation Chromatography | 169 |
| Mode of Operation | 169 |
| Gel Filtration Media | 172 |
| Preparation of the Medium | 176 |
| Preparation of the Column | 178 |
| Determination of the Void Volume | 185 |
| Sample Application and Chromatography | 187 |
| Experimental | 189 |
| Silanization of a Column | 189 |
| Separation of Blue Dextran 2000® and Bromophenol Blue Using Sephadex G-25 | 190 |

| | | |
|-------------------|--|------------|
| | References | 192 |
| Chapter 6. | Electrophoresis | 194 |
| | Ion Movement in an Electric Field | 194 |
| | Acrylamide Gel Electrophoresis | 195 |
| | Electrophoretic Process | 201 |
| | Disc Gel Electrophoresis | 204 |
| | SDS Acrylamide Gel Electrophoresis | 206 |
| | Variations of Acrylamide Gel Electrophoresis | 209 |
| | Slab Gel Electrophoresis | 209 |
| | Agarose-Acrylamide Gels | 210 |
| | Two-Dimensional Gel Electrophoresis | 211 |
| | Detection of Macromolecules Separated by Electrophoresis | 212 |
| | Coomassie Brilliant Blue Staining | 213 |
| | Fluorescent Staining Techniques | 213 |
| | Specific Enzyme Visualization | 214 |
| | Miscellaneous Staining Procedures | 216 |
| | Detection of Radioactive Macromolecules | 216 |
| | Experimental | 219 |
| | Zone Electrophoresis | 219 |
| | Zone Electrophoresis of Fluorescamine Labeled Proteins | 227 |
| | Disc Gel Electrophoresis of Lactate Dehydrogenase Using Nitroblue Tetrazolium for Enzyme Visualization | 228 |
| | References | 232 |
| Chapter 7. | Affinity Chromatography | 234 |
| | Chromatographic Matrix | 238 |
| | Ligand Selection | 239 |
| | Linkage of Ligand and Supporting Matrix | 240 |
| | Absorbent Derivatives | 244 |
| | Chromatography | 246 |
| | References | 254 |
| Chapter 8. | Immunochemical Techniques | 256 |
| | Antibody Structure | 256 |
| | Antibody Formation | 259 |

| | |
|---|------------|
| Practical Aspects of Antibody Production | 264 |
| Antigen | 264 |
| Adjuvants | 265 |
| Animals, Dose, and Route of Inoculation | 266 |
| Response to Inoculation | 267 |
| Serum Collection and Preparation | 270 |
| Reaction of Macromolecular Antigens and | |
| Antibodies in Solution | 274 |
| Antigen-Antibody Reactions in Gels | 277 |
| Immunoelectrophoresis | 283 |
| Use of Antibodies for Specific, High Resolution | |
| Assay of Proteins | 285 |
| Safety | 286 |
| Choice of Radioactive Label for the | |
| Antigen | 286 |
| Direct Immunoprecipitation of Antigens | 288 |
| Demonstration of de novo Protein | |
| Synthesis | 288 |
| Demonstration of an Inactive Form of an | |
| Enzyme | 290 |
| ³⁵ S-Methionine Synthesis | 293 |
| Radioimmunoassay | 295 |
| ¹²⁵ I Labeling Procedures | 297 |
| Standardization of Radioimmunoassays | 299 |
| Experimental | 304 |
| Preparation of Avidin-immune Serum | 304 |
| Quantitative Precipitation of an Antigen | 305 |
| Double Diffusion of Avidin and Avidin- | |
| immune Serum in Ouchterlony Plates | 307 |
| References | 307 |
| Chapter 9. Centrifugation | 309 |
| Relative Centrifugal Force | 309 |
| Desk Top Clinical Centrifuges | 311 |
| Highspeed Centrifuges | 311 |
| The Ultracentrifuge | 312 |
| Drive and Speed Control | 317 |
| Temperature Control | 320 |
| Vacuum System | 320 |
| Rotors | 320 |
| Sedimentation Coefficients | 323 |

| | |
|---|------------|
| The Density Gradient | 326 |
| Sedimentation Velocity or Zone | |
| Centrifugation | 326 |
| Sedimentation Equilibrium or Isopycnic | |
| Centrifugation | 327 |
| Gradient Fractionation | 331 |
| Refractometric Determination of | |
| Concentration | 334 |
| Sedimentation Analysis in a Preparative | |
| Ultracentrifuge | 336 |
| Specific Design of a Density Gradient | 339 |
| Large Scale Centrifugation in Zonal Rotors | 346 |
| Experimental | 347 |
| Isolation of Mitochondria, Proplastids, and | |
| Glyoxysomes on Linear and Stepped | |
| Sucrose Gradients | 347 |
| References | 352 |
| Chapter 10. Protein Purification | 355 |
| Development of an Assay | 355 |
| Selection of a Source from which | |
| Macromolecule may be Isolated | 357 |
| Method of Solubilization | 358 |
| Osmotic Lysis | 358 |
| Grinding | 359 |
| Blenders | 361 |
| Ultrasonic Waves | 362 |
| Presses | 363 |
| Removal of Proteins from Subcellular | |
| Components | 363 |
| Stabilization | 363 |
| pH | 365 |
| Degree of Oxidation | 365 |
| Heavy Metal Contamination | 366 |
| Medium Polarity and Ionic Strength | 367 |
| Protease or Nuclease Contamination | 367 |
| Temperature | 368 |
| Isolation and Concentration | 368 |
| Differential Solubility | 370 |
| Dialysis and Concentration | 378 |
| Ion Exchange Chromatography | 385 |

| | |
|---|-----|
| Conductance Measurement of Ionic Strength | 389 |
| Electrophoresis and Molecular Sieve Chromatography | 390 |
| Criteria of Purity | 390 |
| Experimental | 391 |
| Purification of Acid Phosphatase from Wheat Germ | 391 |
| Establishment of Appropriate Assay Conditions for Acid Phosphatase | 398 |
| Determination of the Michaelis Constant of Acid Phosphatase for <i>p</i> -Nitrophenyl Phosphate | 402 |
| Construction of a Purification Table | 403 |
| References | 404 |

| | | |
|----------------------|--|------------|
| Appendix I. | Concentration of Acids and Bases: Common Commercial Strengths | 407 |
| Appendix II. | International Scale (1936) of Refractive Indexes of Sucrose Solutions at 20°C | 408 |
| Appendix III. | Density at 25°C of CsCl Solution as a Function of Refractive Index | 409 |
| Appendix IV. | Periodic Table of Elements | 410 |
| Index | | 413 |

Chapter 1

Potentiometric Techniques

Most of the chemical reactions that comprise a living organism are profoundly influenced by hydrogen ion concentration. So important is this characteristic that multicelled organisms have evolved a variety of sophisticated methods to maintain the solutions in which their cells are bathed within rigid limits of hydrogen ion concentration. The same care exercised by living organisms to maintain acceptable hydrogen ion concentrations must be duplicated in the laboratory if meaningful insights are to be gained into the functioning of organisms and their components. The subsequent discussion employs the Brønsted-Lowry definition of acids and bases: an acid is a compound that donates protons and a base is one that accepts protons. This definition may also be formulated as an acid dissociating into a base and a proton:



Therefore, HCl would be considered an acid and Cl^- would be its conjugate base.

| Acid | Conjugate Base |
|--------------------------|---------------------------|
| HCl | Cl^- |
| CH_3COOH | CH_3COO^- |
| H_2CO_3 | HCO_3^- |
| HCO_3^- | CO_3^{2-} |
| NH_4^+ | NH_3 |

Acids and bases can be classified as strong or weak depending on the extent to which they ionize. A strong acid is one for which reaction 1

proceeds far to the right; that is, the acid is essentially totally ionized. For example, the hydrogen ion concentration of a $0.01M$ solution of the strong acid HCl is $0.01M$ because all of the acid is dissociated. On the other hand, a weak acid (such as acetic, boric, or carbonic) is one for which reaction 1 does not proceed significantly to the right.

pH CALCULATIONS

A Danish chemist, S. P. L. Sorensen, proposed a convenient notation for the hydrogen ion concentration of a solution. He defined the negative log of the hydrogen ion concentration as pH.

$$pH = -\log [H^+] \quad (2)$$

Therefore, for a $0.01M$ solution of HCl ,

$$\begin{aligned} pH &= -\log [10^{-2}] \\ &= 2.0 \end{aligned}$$

The pH values most often encountered in biochemistry range from 4 to 11. Figure 1-1 depicts the relationship of pH to acidity and basicity or alkalinity.

It is clear that the pH of a $10^{-2}M$ solution of a strong acid is 2.0, but the

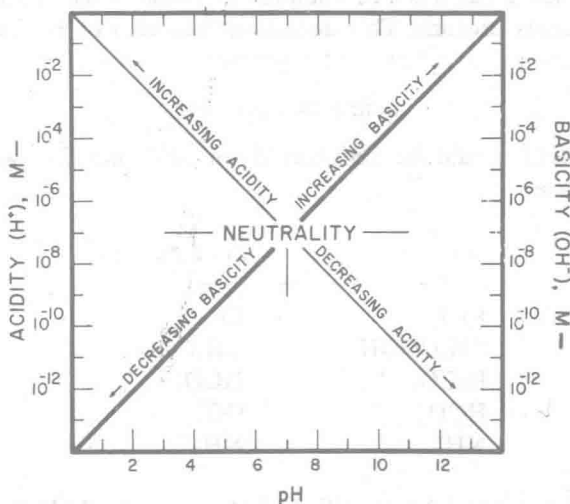
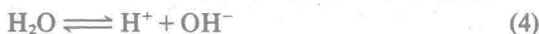


Figure 1-1. The relationship of acidity and basicity or alkalinity of a solution to its hydrogen ion and hydroxyl ion concentrations.

pH of a 0.01M solution of a strong base (reaction 3) is less obvious.



The hydroxyl ion concentration can be related to the hydrogen ion (H^+), or more accurately hydronium ion (H_3O^+), concentration by considering the dissociation of water:



The equilibrium constant equation for this reaction is

$$K_{eq} = \frac{[\text{H}^+][\text{OH}^-]}{[\text{H}_2\text{O}]} \quad (5)$$

and thus

$$K_{eq}[\text{H}_2\text{O}] = [\text{H}^+][\text{OH}^-] \quad (6)$$

Since the concentration of water remains about constant throughout the ionization process this term can be combined with the equilibrium constant, generating a new constant, K_w :

$$K_w = [\text{H}^+][\text{OH}^-] \quad (7)$$

For pure water at 25°C both the hydronium and hydroxyl ion concentrations are equal to $1 \times 10^{-7}M$. Therefore,

$$\begin{aligned} K_w &= (1 \times 10^{-7})(1 \times 10^{-7}) \\ &= 1 \times 10^{-14} \end{aligned}$$

Since in aqueous solution, the product of the hydronium and hydroxyl ion concentrations must remain constant at $1 \times 10^{-14}M$, an increase in one term of equation 7 requires a corresponding decrease in the other term. Therefore, a 0.01M NaOH aqueous solution has a hydrogen ion concentration of

$$\begin{aligned} [\text{H}^+] &= \frac{K_w}{[\text{OH}^-]} \\ &= \frac{10^{-14}}{10^{-2}} \\ &= 10^{-12} \end{aligned} \quad (8)$$

and

$$\text{pH} = 12$$

Weak acids, by definition, are only partially ionized in aqueous solution.



The concentration of each species at equilibrium may be calculated from the dissociation constant of the acid as described by the equation

$$K_a = \frac{[H^+][A^-]}{[HA]} \quad (10)$$

Rearrangement of equation 10 gives

$$[H^+] = \frac{[HA]K_a}{[A^-]} \quad (11)$$

If the negative logarithms of both sides of equation 11 are taken,

$$-\log [H^+] = (-\log K_a) + \left(-\log \frac{[HA]}{[A^-]} \right) \quad (12)$$

or

$$pH = pK_a + \log \frac{[A^-]}{[HA]} \quad (13)$$

In more general terms,

$$pH = pK_a + \log \frac{[\text{conjugate base}]}{[\text{undissociated acid}]} \quad (14)$$

This equation is known as the Henderson-Hasselbach equation. If

$$[A^-] = [HA]$$

then

$$pH = pK_a \quad (15)$$

Many handbooks list dissociation constants (the equilibrium constant for the dissociation of acid into a proton and its conjugate base) as pK_a 's.

Thus far it has been assumed that the molar concentration of any given ion is also its effective or active concentration. This, however, is true only at very low ion concentrations. As the number of ions in a given volume increases, the probability of ionic interactions also increases. These interactions tend to impede the movement of ions and hence decrease their effective concentration or activity. Activity is related to molar concentration by a normalization factor or activity coefficient

$$a_i = f_i [i] \quad (16)$$

where a_i is the activity of ionic species i , and f_i is the activity coefficient. When ions are separated from one another (at low concentration) f_i approaches unity; f_i decreases as the concentration of i increases. The distinction between the activity and molar concentration of an ion is significant because all potentiometric measurements of hydrogen ion concentration yield hydrogen ion activity, not concentration.