

**amino
acid
determination**

methods
and
techniques

second edition, revised and expanded

edited by
S. BLACKBURN

AMINO ACID DETERMINATION

Methods and Techniques

SECOND EDITION, REVISED AND EXPANDED

MARCEL DEKKER, INC. New York and Basel

Library of Congress Cataloging in Publication Data

Main entry under title:

Amino acid determination.

Includes bibliographies and indexes.

1. Amino acids--Analysis. I. Blackburn, Stanley.

Amino acid determination. QD431.A66 1978 547'.75 78-15680
ISBN 0-8247-6349-1

Copyright ©1978 by Marcel Dekker, Inc. All rights reserved

Neither this book nor any part may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopying, microfilming, and recording, or by any information storage and retrieval system, without permission in writing from the publisher.

Marcel Dekker, Inc.

270 Madison Avenue, New York, New York 10016

Current printing (last digit):

10 9 8 7 6 5 4 3 2 1

Printed in the United States of America

PREFACE

Methods of amino acid determination, which are of prime importance in biochemical studies, may be considered to have come of age some 10 or 20 years ago. These methods are nevertheless subject to continuing change and improvement. Techniques have developed rapidly, and the advantages and capabilities of the different procedures are now clearly understood. Continuing improvements in their speed, sensitivity, and versatility are being made which give reliable information on amino acid composition from even small amounts of protein or peptide. Fluorometric methods of detection may permit further increases in sensitivity to be achieved. Since the appearance of the first edition of this monograph, the automatic analyzer has maintained and strengthened its preeminent position in the field of amino acid determination. The instrument is reviewed in the present book by Dr. G. W. Robinson, who is actively involved in development and use of the analyzer. The application of computer techniques to the control of automatic analyzers is developing rapidly as is the computation of results obtained from them; this topic is reviewed by workers in this field.

Striking developments in gas chromatographic techniques have taken place in parallel with those in automatic analyzer methods; procedures for the separation of enantiomers of amino acids are particularly important. Use of both analytical techniques in the study of lunar material reflects their importance. The increase in sensitivity of automatic analyzer and other methods and the consequent reduction in the size of analytical samples has meant that increasing care is required to avoid contamination of these samples.

Marked advances in hydrolytic techniques mean that sources of error arising during hydrolysis are minimized. Determination of all the common protein amino acids using a single hydrolyzate is now feasible.

The present volume, while in no sense a practical manual, assembles authoritative accounts of the various procedures and techniques, emphasizing the practical application of means of determination of amino acids. Biochemists and analysts can thus select methods appropriate to their research problems. The biological aspect of amino acid determination is stressed in the description of the analysis of physiological fluids.

My thanks are due to the contributors to the present volume for their ready and enthusiastic cooperation.

S. Blackburn

CONTRIBUTORS

S. Blackburn, Kepstorn Road, Leeds, England

Anthony M. C. Davies, Agricultural Research Council's Food Research Institute, Norwich, England

Klaus Hempel, Institut für Medizinische Strahlenkunde der Universität Würzburg, Würzburg, Federal Republic of Germany

M. W. Johnson, Department of Virus Research, John Innes Institute, Norwich, England

Hans-Walter Lange, Institut für Medizinische Strahlenkunde der Universität Würzburg, Würzburg, Federal Republic of Germany

Norbert Lustenberger,* Institut für Medizinische Strahlenkunde der Universität Würzburg, Würzburg, Federal Republic of Germany

George W. Robinson,** Department of Biochemistry, University of Kentucky, Lexington, Kentucky

Roger Stansfield, Agricultural Research Council's Food Research Institute, Norwich, England

André C. Van Steirteghem,† Clinical Pathology Department, Clinical Center, National Institutes of Health, Bethesda, Maryland

Donald S. Young,‡ Clinical Pathology Department, Clinical Center, National Institutes of Health, Bethesda, Maryland

* Current affiliation: Medizinische Hochschule Hannover, Department of Nephrology, Hannover, Federal Republic of Germany

** Present address: 333 Grosvenor Avenue, Lexington, Kentucky 40508

† Current affiliation: Clinical Chemistry Service, Vrije Universiteit Brussel, Universitair Ziekenhuis Sint-Pieter, Brussels, Belgium

‡ Current affiliation: Department of Laboratory Medicine, Mayo Clinic and Foundation, Rochester Minnesota

CONTENTS

Preface	iii
Contributors	v
1. Amino Acid Analysis: An Important Technique	1
<i>S. Blackburn</i>	
I. Introduction	1
II. Development of Methods of Determination	3
References	6
2. Sample Preparation and Hydrolytic Methods	7
<i>S. Blackburn</i>	
I. Sample Preparation	8
II. Acid Hydrolysis	9
III. Hydrolysis with Sulfonic Acids	21
IV. Alkaline Hydrolysis	25
V. Enzymatic Hydrolysis	28
VI. Avoidance of Contamination	32
References	35
3. Development of Ion-Exchange Techniques	39
<i>George W. Robinson</i>	
4. Factors Affecting Separation of Amino Acids on Ion-Exchange Columns	47
<i>George W. Robinson</i>	
I. General Characteristics of Separation	48
II. Theoretical Aspects of Separation	52
III. Parameters Involved in Separation	56
References	66
5. Buffers for Ion-Exchange Columns	69
<i>George W. Robinson</i>	
I. Classification of Systems	70
II. Analysis of Buffer Variables	73
III. Examples of Buffer Systems	77
IV. Ligand-Exchange Chromatography	82
References	85

6. Detection Systems for Amino Acids in Ion-Exchange Column Effluents	89
<i>George W. Robinson</i>	
I. General Description	90
II. Reagents for Detecting Amino Acids	90
III. Segmented and Nonsegmented Analytical Systems	94
IV. Detection and Amplification of Signal	95
V. Computation of Results	97
VI. Accuracy and Precision	98
References	98
7. Automatic Analyzers	101
<i>George W. Robinson</i>	
I. General Types of Automatic Analyzers	101
II. Speed and Sensitivity	102
III. Practical Modifications	104
References	107
8. Gas Chromatographic Methods of Amino Acid Analysis	109
<i>S. Blackburn</i>	
Part One: Determination of Amino Acids	110
I. Introduction	110
II. Detectors	113
III. Choice of a Suitable Derivative	116
IV. Support and Stationary Phase	125
V. Column Separation of N-Trifluoroacetyl Amino Acid n-Butyl Esters	129
VI. N-Heptafluorobutyryl Derivatives	134
VII. Trimethylsilyl Derivatives	138
VIII. Amino Acid Phenylthiohydantoins	142
IX. Accuracy and Reproducibility of Gas Chromatographic Determination	147
X. Discussion	152
Part Two: Separation of Amino Acid Enantiomers	154
I. Introduction	154
II. Conversion of Enantiomers to Diastereoisomers	154
III. Use of Optically Active Stationary Phases	158
IV. Structural Factors Affecting Separation of Enantiomers	165
V. Quantitative Determination of Enantiomers	171
VI. Conclusions	172
VII. Applications of Enantiomer Separation	173
Addendum	179
References	183

9. Application of Computers to Amino Acid Analysis	189
<i>Roger Stansfield, Anthony M. C. Davies, and M. W. Johnson</i>	
I. Introduction	190
II. Equipment	192
III. Peak-Area Estimation	196
IV. Peak Identification	204
V. Calculation of Results	206
VI. Commercially Available Systems	210
VII. Other Computer Techniques	212
VIII. Conclusions	215
References	217
10. Applications of Amino Acid Analysis: Miscellaneous Procedures	221
<i>S. Blackburn</i>	
I. Estimation of Unusual Amino Acids	222
II. Separation of Amino Acid Enantiomers on Ion-Exchange Resins	227
III. Determination of Tryptophan and Cystine	229
IV. Analysis of Foods	235
V. Gas Chromatography-Mass Spectrometer Techniques	241
VI. X-Ray Crystallographic Methods	246
VII. Use of 1-Dimethylaminonaphthalene-5- sulfonyl Amino Acids	250
References	256
11. Amino Acids in Physiological Fluids	261
<i>André C. Van Steirteghem and Donald S. Young</i>	
I. Introduction	263
II. Methods for Qualitative and Quantitative Analysis of Amino Acids in Physiological Fluids	264
III. Amino Acids in Body Fluids	275
IV. Laboratory Factors	283
V. Physiological Factors	285
VI. Diseases	291
VII. Inborn Errors of Metabolism	301
VIII. Influence of Medications	307
References	309
12. The Pyridoxal Method: Total Amino Acid Analysis in the Micro- to Picomole Range After Preliminary Derivatization with Pyridoxal	319
<i>Klaus Hempel, Hans-Walter Lange, and Norbert Lustenberger</i>	
I. Introduction	320
II. Pyridoxyl Amino Acids: Synthesis and Properties	320

III. Detection	323
IV. Chromatographic Separation and Quantitation	329
V. Applications	331
References	335
13. Present Status and Future Development of Amino Acid Analysis	337
<i>S. Blackburn</i>	
I. Present Status	337
II. Future Developments	340
References	344
Author Index	345
Subject Index	363

CHAPTER 1

Amino Acid Analysis: An Important Technique

S. Blackburn

Leeds, England

I. Introduction	1
II. Development of Methods of Determination	3
References	6

I. INTRODUCTION

The importance of amino acid analysis nowadays needs no emphasis; indeed, it is almost accepted as commonplace. None of the remarkable advances of the last decades in protein and enzyme structure would have been possible without reliable techniques of amino acid determination. If we had to use specific procedures to determine each amino acid, the analysis of a protein would be very tedious and time consuming, as was Brand's analysis of β -lactoglobulin in 1946 [1]. Modern techniques, which are largely chromatographic in nature, owe much to the studies of A. J. P. Martin and R. L. M. Synge and

were developed to their present advanced state by S. Moore and W. H. Stein (see Chaps. 3 to 8).

These techniques form an integral part of almost all biochemical structure investigations. Amino acid analysis bears a relationship to the chemistry of proteins and peptides similar to that which elementary analysis bears to the chemistry of simpler organic molecules. Amino acid analysis, for example, gives evidence as to the purity of proteins. Thus, an analysis of streptococcal proteinase, referred to a sample dried to constant weight in vacuo over P_2O_5 , accounted for 98% of the weight and 99% of the nitrogen of the sample [2]. Amino acid analysis can even be used to determine the amounts of a protein or peptide being studied. Hodges and Merrifield [3] adopted this approach in an investigation of the effects of a tyrosine residue at position 120 of ribonuclease on the enzyme's properties. To avoid problems of determining dry weights of proteins and peptides, all quantities of RNase, RNase 1-118, and synthetic COOH-terminal peptides were determined from amino acid analyses. The mean of the molar ratios of all accurately measurable amino acids in the acid hydrolyzate was used to calculate the concentration of the protein or peptide. Accuracy of determination is of importance in studying newly isolated proteins. When a protein of unknown composition is being analyzed, the key calculation is the correlation of its molecular weight with integral numbers of amino acid residues which are present in small molar proportions. Accuracy is essential for this type of correlation.

In investigations not involving large proteins or peptides, sophisticated techniques may not always be required. Shipolini et al. [4], when studying phospholipase A_2 from bee venom, found that for small peptides, qualitative analyses by the method of complete dansylation* gave satisfactory results. The residue of amino acids after NH_2 -terminal analysis was completely dansylated and the resulting mixture of dansyl amino acids separated by thin layer chromatography. Composition of the mixture was assessed by visual comparison of the intensity of fluorescence of separated spots.

*Dansyl is a contraction for dimethylaminonaphthalenesulfonyl.

Although amino acid analysis of a protected nonapeptide obtained by the solid phase method gave the expected ratios of the constituent amino acids, similar correct ratios were obtained from analyses of all the fractions prior to crystallization and even of the crude oil [5]. Similar results were obtained by Beacham et al. [6], who observed that the amino acid composition of acid or enzymatic hydrolyzates of column-purified peptides agreed closely with that found for the crude product. Amino acid analyses in agreement with theory are thus a necessary but not a sufficient criterion of purity. Exceptionally, the amino acid sequence and three-dimensional structure of the main component of sperm whale myoglobin preparations was determined several years before a direct amino acid analysis was made [7]. The amino acid analysis [8], however, then revealed small differences in composition between the closely similar components of these myoglobin preparations.

II. DEVELOPMENT OF METHODS OF DETERMINATION

The assured status of the methods in current use may be readily seen by comparing the first edition of this book [9] with the present one and considering changes that have occurred in the interval between the publication of the two. The earlier edition gave a short description of the historical development of amino acid techniques and the development of ion-exchange resin techniques and the automatic analyzer.

Today the techniques of amino acid determination are well established, and a description, even brief, of early methods such as chromatography on starch columns is of little practical interest. Attention is therefore concentrated on techniques which are currently in use or are likely to assume importance in the future. Pride of place among methods of determination in use today is taken by the automatic analyzer, which is still in principle that devised by Moore and Stein. The manual ion-exchange method is hardly ever used. Amino

acid determination by the automatic analyzer gives results of high precision and accuracy. This aspect of the determination has been discussed by Moore [10].

The automatic analyzer has been refined and modified over the years so that an originally sophisticated instrument is now even more sophisticated and powerful. Novel features have been incorporated in recently designed analyzers, the Rank-Hilger Chromaspek developed in Britain, for example, incorporates a completely new device for applying a buffer gradient to the column of ion-exchange resin. Fluorimetric amino acid analyzers have been developed which use fluorecamine [11] or o-phthalaldehyde and 2-mercaptoethanol [12] to estimate the amino acids in the column effluent. These refinements and the improvements in methodology are described in Chaps. 3 to 8. Computer techniques used in conjunction with automatic analyzers are described in Chap. 9. The preparation of fluorescent derivatives of amino acids and their separation on ion-exchange columns is described in Chap. 12.

The gas chromatographic method has failed to displace the amino acid analyzer as the principal means of determining amino acids in spite of the large volume of work published on the topic. The separation and estimation of amino acid enantiomers by gas chromatography, however, may well assume a dominant role. The techniques of amino acid determination have been improved to such an extent that amounts of amino acid of the order of 1 ng can be estimated. The analysis of lunar samples is a case where the great sensitivity of the methods, both ion-exchange resin and gas chromatographic, were exploited.

Examination of biological samples and physiological fluids on necessarily very small amounts of material is becoming important in the diagnosis of diseased states and altered metabolic conditions. Accurate and sensitive assays of amino acids and metabolites are a prerequisite for the study of biological materials. These developments are reviewed in Chap. 11. Methods involving the use of gas chromatography and the mass spectrometer in conjunction have been

developed for the analysis of amino acids at the nanogram and sub-nanogram level and are described in Chap. 10.

Methods which involve the separation of derivatives, often those used in determining the NH_2 -terminal amino acids of peptides, on paper or thin-layer chromatograms seem capable of providing accurate results. In practice, however, only rarely are they widely adopted, and many are used only by the authors who originally described them. In spite of its historical importance, paper chromatographic determination of free amino acids is now scarcely used in a quantitative or semiquantitative way, at least in studying pure proteins.

The present study is concerned with methods of amino acid analysis which are of practical use to the biochemist and analyst. Described in less detail are techniques which, although yielding data on amino acid composition, are not primarily designed with this end in view. The accuracy with which the amino acid composition of proteins can be determined depends not only on the instrumentation used to determine the amino acids themselves but also on the methods used to hydrolyze the proteins into free amino acids. Techniques for the hydrolysis of peptides and proteins have improved greatly over the last few years. The method using HCL as the hydrolytic agent has been modified by the addition of agents to minimize destruction of amino acids, whereas alkaline hydrolysis has been modified to give accurate results for tryptophan. Hydrochloric acid as the hydrolytic agent has been replaced in several techniques by sulfonic acids, and a method of this type developed by T. J. Liu and colleagues allows the determination of all the common protein amino acids in a single hydrolyzate. (These methods are discussed in Chap. 2.)

The sensitivity of the newer analytical procedures is such that it is possible to determine extremely small amounts of amino acid. Under these conditions, unless stringent precautions are taken the level of contaminating amino acids in reagents, for example, may be of the same order of magnitude as the quantity of amino acid it is desired to determine. These precautions are described in Chap. 2.

REFERENCES

1. E. Brand, *Ann. N. Y. Acad. Sci.*, **47**, 187 (1946).
2. T.-Y. Liu, N. P. Neumann, S. D. Elliott, S. Moore, and W. H. Stein, *J. Biol. Chem.*, **238**, 251 (1963).
3. R. S. Hodges and R. B. Merrifield, *Int. J. Peptide Protein Res.*, **6**, 397 (1974).
4. R. A. Shipolini, G. L. Callewaert, R. C. Cottrell, and C. A. Vernon, *Eur. J. Biochem.*, **48**, 465 (1974).
5. J. Meienhofer and Y. Sano, *J. Amer. Chem. Soc.*, **90**, 2996 (1968).
6. J. Beacham, G. Dupuis, F. M. Finn, H. T. Storey, C. Yanaihara, N. Yanaihara, and K. Hofmann, *J. Amer. Chem. Soc.*, **93**, 5526 (1971).
7. A. B. Edmundson, *Nature*, **205**, 883 (1965).
8. M. H. Garner, W. H. Garner, and F. R. N. Gurd, *J. Biol. Chem.*, **249**, 1513 (1974).
9. S. Blackburn, *Amino Acid Determination: Methods and Techniques*, New York, Marcel Dekker, 1968.
10. S. Moore, *Chemistry and Biology of Peptides*, Ann Arbor, Michigan, Ann Arbor Science Publ., 1972, p. 629.
11. S. Udenfriend, S. Stein, P. Bohlen, W. Dairman, W. Leimgruber, and M. Weigele, *Science*, **178**, 871 (1972).
12. M. Roth and A. Hampai, *J. Chromatogr.*, **83**, 353 (1973).

CHAPTER 2

Sample Preparation and Hydrolytic Methods

S. Blackburn

Leeds, England

I. Sample Preparation	8
II. Acid Hydrolysis	9
A. Destruction of Amino Acids	10
B. Racemization of Amino Acids	19
C. Slow Liberation of Amino Acids	21
III. Hydrolysis with Sulfonic Acids	21
IV. Alkaline Hydrolysis	25
V. Enzymatic Hydrolysis	28
VI. Avoidance of Contamination	32
A. Hands	32
B. Glassware	33
C. Reagents	33
D. Water	34
References	35