amino acid determination

methods
and
techniques

second edition, revised and expanded

edited by
S. BLACKBURN

AMINO ACID DETERMINATION

Methods and Techniques

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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

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*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 Zeikenhuis Sint-Pieter, Brussels, Belgium

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

CONTENTS

CHAPTER 1

Amino Acid Analysis: An Important Technique

S. Blackburn

Leeds, England

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 propertions. 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 phospolipase 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 dimethylaminonapthalenesulfonyl.

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 fluorescamine [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 subnanogram 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 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