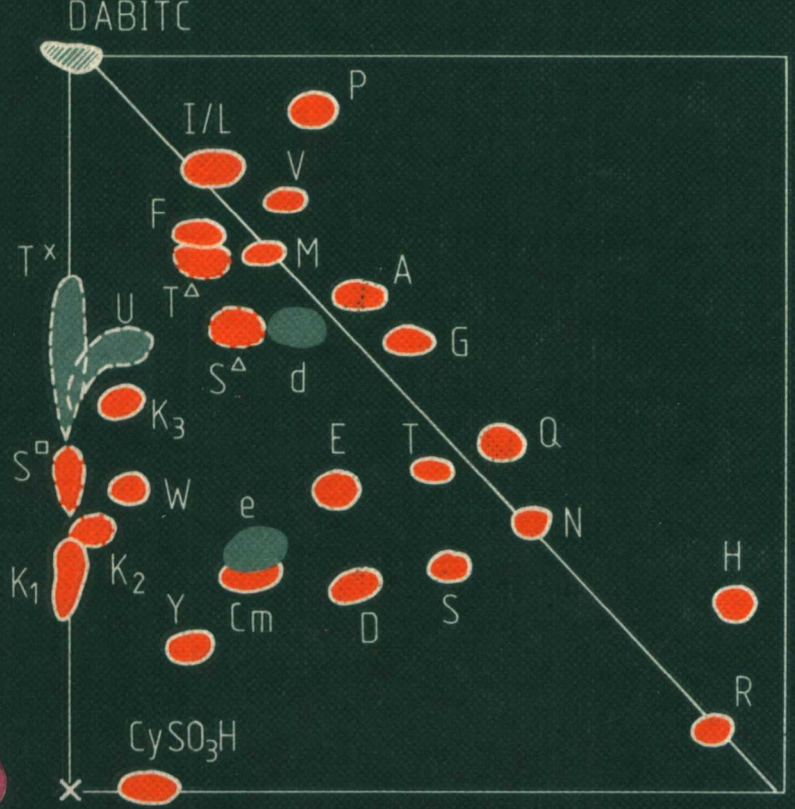
Advanced Methods in Protein Microsequence Analysis

Edited by Brigitte Wittmann-Liebold
Johann Salnikow, and Volker A. Erdmann



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With 165 Figures

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Cover illustration:
Separation of DABTH-amino acid derivatives two-dimensional polyamide thin-layer sheets (see also Fig. 2 on p. 85)

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AMONS, R. 352
ASHMAN, K. 219
BAUW, G. 179
BEEUMEN, J. VAN 256
BENNETT, J.C. 208
BERGMAN, T. 45
BEYREUTHER, K. 276
BHOWN, A.S. 208
BLÖCKER, H. 387
BORCHART, U. 91
BOULANGER, Y. 291
BRANDT, W.F. 161
BROCKMÖLLER, H.-J. 34
BUSE, G. 340
CARLQUIST, M. 45
CHANG, JUI-YOA 56, 265
CRABB, J.W. 64
DAMME, J. VAN 179
DIGWEED, M. 364
ECKART, K. 403
ERDMANN, V.A. 364
FRANK, RAINER 149
FRANK, RONALD 387
FRIEDRICH, J. 226
GAUSEPOHL, H. 149
GIEGE, R. 291
GOTTO, A.M., Jr. 320
HENSCHEN, A. 244
HENSEL, S. 340
HIRANO, H. 77
HOLT, C. VON 161
JÖRNVALL, H. 45
KALKKINEN, N. 194
KAMP, R.M. 8, 21, 34
KERN, D. 291
KIMURA, M. 77
KNECHT, R. 56
KRAUHS, E. 316
KUHN, C.C. 64
LITTLE, M. 316
MACHLEIDT, W. 91
MAIER, G. 316
MEINECKE, L. 126, 340
MEJDOUB, H. 291
MONTAGU, M. VAN 179
PIELER, T. 364
PONSTINGL, H. 316
POWNALL, H.J. 320
PRINZ, H. 276
PUYPE, M. 179
REIMANN, F. 118
REINBOLT, J. 291
REUMKENS, J. 340
RITONJA, A. 91
SALNIKOW, J. 108
SCHULZE-GAHMEN, U. 276
SPIESS, J. 302
STEFFENS, G.C.M. 340
STEFFENS, G.J. 340
TROSIN, M. 149
TSCHESCHE, H. 126
VANDEKERCKHOVE, J. 179
WITTMANN-LIEBOLD, B. 77, 118
YANG, CHAO-YUH 320
YANG, TSEMING 320

Contents

Introduction 1

Chapter 1
Separation and Amino Acid Analysis of Proteins and Peptides for Microsequencing Studies

1.1 Separation of Peptides
R. M. KAMP (With 6 Figures) 8

1.2 High Performance Liquid Chromatography of Proteins
R. M. KAMP (With 4 Figures) 21

1.3 Two-Dimensional Polyacrylamide Gel Electrophoresis in Stamp-Sized Gels
H.-J. BROCKMÖLLER and R. M. KAMP (With 6 Figures) 34

1.4 Amino Acid Analysis by High Performance Liquid Chromatography of Phenylthiocarbamyl Derivatives
T. BERGMAN, M. CARLQUIST, and H. JÖRNVALL (With 3 Figures) 45

1.5 High Sensitivity Amino Acid Analysis Using DABS-Cl Precolumn Derivatization Method
R. KNECHT and JUI-YOA CHANG (With 2 Figures) 56

Chapter 2
Manual and Solid-Phase Microsequencing Methods

2.1 Modern Manual Microsequencing Methods
C. C. KUHN and J. W. CRABB (With 5 Figures) 64

2.2 Manual Microsequence Determination of Proteins and Peptides with the DABITC/PITC Method
B. WITTMANN-LIEBOLD, H. HIRANO, and M. KIMURA (With 3 Figures) 77

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2.3 Solid-Phase Microsequencing: Procedures and Their Potential for Practical Sequence Analysis
W. MACHLEIDT, U. BORCHART, and A. RITONJA
(With 8 Figures) 91

2.4 Automated Solid-Phase Microsequencing Using DABITC, On-Column Immobilization of Proteins
J. SALNIKOW (With 2 Figures) 108

Chapter 3
Gas-Phase and Radio-Sequence Analysis

3.1 Gas-Phase Sequencing of Peptides and Proteins
F. REIMANN and B. WITTMANN-LIEBOLD (With 1 Figure) ... 118

3.2 Water Contents and Quality Criteria of Microsequencing Chemicals. Preliminary Results of a Reevaluation
L. MEINECKE and H. TSCHESCHE (With 9 Figures) 126

3.3 An Improved Gas-Phase Sequenator Including On-Line Identification of PTH Amino Acids
H. GAUSEPOHL, M. TROSIN, and RAINER FRANK
(With 7 Figures) 149

3.4 Amino-Acid Composition and Gas-Phase Sequence Analysis of Proteins and Peptides from Glass Fiber and Nitrocellulose Membrane Electro-Blots
W. F. BRANDT and C. VON HOLT (With 14 Figures) 161

3.5 Protein Blotting from Polyacrylamide Gels on Glass Microfiber Sheets: Acid Hydrolysis and Gas-Phase Sequencing of Glass-Fiber Immobilized Proteins
J. VANDEKERCKHOVE, G. BAUW, M. PUYPE,
J. VAN DAMME, and M. VAN MONTAGU (With 8 Figures) ... 179

3.6 Radio-Sequence Analysis; an Ultra-Sensitive Method to Align Protein and Nucleotide Sequences
N. KALKKINEN (With 4 Figures) 194

Chapter 4
Phenylthiohydantoin Identification, On-Line Detection, Sequences Control, and Data Processing

4.1 Conversion of Anilinothiazolinone to Phenylthiohydantoin Derivatives and Their Separation by High Pressure Liquid Chromatography
A. S. BHOWN and J. C. BENNETT (With 7 Figures) 208

4.2 The Use of On-Line High Performance Liquid Chromatography for Phenylthiohydantoin Amino-Acid Identification
K. ASHMAN (With 2 Figures) 219

4.3 Device Control, Data Collection, and Processing in Protein Micro-Sequencing and Amino-Acid Analysis
J. FRIEDRICH (With 5 Figures) 226

Chapter 5
Analysis of Cysteine Residues in Proteins

5.1 Analysis of Cyst(e)ine Residues, Disulfide Bridges, and Sulfhydryl Groups in Proteins
A. HENSCHEN (With 2 Figures) 244

5.2 Identification of the Heme-Binding Cysteines in Cytochromes c Without Radioactive Labeling
J. VAN BEEUMEN (With 4 Figures) 256

5.3 Micro-Isolation of Polypeptides Precolumn Labeled with Hydrophobic Chromophore
JUI-YOA CHANG (With 7 Figures) 265

Chapter 6
Methods of Analyzing Protein Conformation

6.1 Synthetic Immunogens for Secondary Structure Assignment: Conformational Sequencing of Proteins with Antipeptide Antibodies
K. BEYREUTHER, H. PRINZ, and U. SCHULZE-GAHMEN (With 7 Figures) 276

6.2 Aspartyl-tRNA Synthetase-Induced Aspartylation of Proteins: a Fingerprint Approach to Map Accessible Domains in Protein
H. MEJDOUB, D. KERN, R. GIEGE, Y. BOULANGER, and J. REINBOLT (With 2 Figures) 291

Chapter 7
Strategies and Specific Examples of Sequencing Proteins and Peptides

7.1 Strategies of Biochemical Characterization of Hormonal Peptides
J. SPIESS (With 4 Figures) 302

7.2 Use of a Metalloproteinase Specific for the Amino Side of Asp in Protein Sequencing
H. PONSTINGL, G. MAIER, M. LITTLE, and E. KRAUHS 316

7.3 Primary Structure of Rabbit Apolipoprotein A-I. High Performance Liquid Chromatography, PTC-Amino Acid Analysis, and Microsequencing
CHAO-YUH YANG, TSEMING YANG, H.J. POWNALL, and A.M. GOTTO, Jr. (With 10 Figures) 320

7.4 Sequence Analysis of Complex Membrane Proteins (Cytochrome c Oxidase)
G. BUSE, G.J. STEFFENS, G.C.M. STEFFENS, L. MEINECKE, S. HENSEL, and J. REUMKENS (With 5 Figures) 340

7.5 Sequence Studies on the α-, β-, and γ-Chains of Elongation Factor 1 from *Artemia*. Some Practical Notes
R. AMONS (With 6 Figures) 352

Chapter 8
Alternative Sequencing Techniques for Elucidation of Peptide and Protein Structures

8.1 RNA Sequencing
M. DIGWEED, T. PIELER, and V.A. ERDMANN (With 5 Figures) 364

8.2 Synthetic Genes as a Powerful Tool for Protein Structure-Function Analysis
RONALD FRANK and H. BLÖCKER (With 11 Figures) 387

8.3 Generation and Interpretation of Fast Atom Bombardment Mass Spectra of Modified Peptides
K. ECKART (With 6 Figures) 403

Subject Index 415

Introduction

Much of the recent spectacular progress in the biological sciences can be attributed ot the ability to isolate, analyze, and structurally characterize proteins and peptides which are present in cells and cellular organelles in only very small amounts. Recent advances in protein chemistry and in particular the application of new micromethods have led to fruitful advances in the understanding of basic cellular processes. Areas where protein-chemical studies have resulted in interesting discoveries include the peptide hormones and their release factors, growth factors and oncogenes, bioenergetics, proton pumps and ion pumps and channels, topogenesis and protein secretion, molecular virology and immunology, membrane protein analysis, and receptor research. In fact, the key methods are now on hand to unravel many of the major outstanding problems of molecular biology and in particular questions of fundamental interest which relate to developmental biology and specificity in cell-cell interaction.

In this volume we have assembled descriptions of procedures which have recently been shown to be efficaceous for the isolation, purification, and chemical characterization of proteins and peptides that are only available in minute amounts. Emphasis is placed on well-established micromethods which have been tested and found useful in many laboratories by experienced investigators. The chapters are written by specialists, and describe a range of sensitive techniques which can be used by researchers working in laboratories with only modest resources and equipment. The book is also a compilation of experimental protocols which are suitable for use in the laboratory for student courses at the advanced undergraduate and graduate level, as well as for use by researchers who are new to the field of protein microsequence analysis. Furthermore, modifications of some newer manual microsequence methods are described that demonstrate that low picomole amino acid sequence analysis may not always require sophisticated equipment. It is our objective to describe these procedures with sufficient clarity that even researchers without prior experience in protein chemistry and especially without experience in protein microsequence analysis can use these methods. By employing the techniques as described the reader is able to avoid errors that cause substance losses at the isolation stages or diminish sequence information.

However, it is not our intention to cover the whole field of contemporary protein chemistry. Conventional methodology which can be adapted readily to the microscale level is not discussed in this book except selected applications at the

microlevel, e.g., the performance of the main chemical and enzymatic cleavages of proteins. A comprehensive manual of protein chemistry, *Practical Protein Chemistry,* edited by A. Darbre, John Wiley & Sons, Chichester, 1986, has recently been published, and review articles on protein-analytical and physicochemical determinations are covered in the series *Modern Methods in Protein Chemistry,* edited by H. Tschesche, Walter deGruyter, Berlin, 1983 and 1985, these volumes providing information where additional basic knowledge in this field is needed. The many gel electrophoresis techniques which have found wide application in protein analysis are detailed in the recent volumes *Gel Electrophoresis of Proteins,* edited by B. D. Hames and D. Rickwood, IRL Press, Oxford, England, 1981; in *Proteins* edited by J. W. Walker, Humana Press, Clifton, New Jersey, 1984; and in *Two-Dimensional Gel Electrophoresis of Proteins, Methods and Applications,* edited by J. E. Celis and R. Bravo, Academic Press Inc., New York, 1984. Conventional manual sequencing techniques such as the dansyl-Edman degradation for peptides are described in detail elsewhere; therefore only more recent and sensitive manual microsequencing techniques which can be applied for peptides as well as for proteins in the picomole range are included here.

Most methods discussed in this book were demonstrated during the FEBS Advanced Course on Microsequence Analysis of Proteins, held at Berlin (West) in September 1985, and organized by our research groups. This course was followed by an International Symposium on *Novel Techniques in Protein Sequence Analysis* and additional information on micromethods in this book is based on the presentations made at this workshop. At this meeting it became obvious that the demand for highly sensitive and practical methods for basic protein research for a wide range of investigations has increased dramatically. We obtained so many applications for attendance at this course that we could not accept most of these researchers, although they all had urgent reasons for learning these techniques for their present research work. Therefore, we decided to assemble and carefully describe the methods reported or demonstrated at the course so that they might be available to a wider scientific community and especially to young students who will need these techniques for future scientific work. Nowadays, protein analytical methods are not the exclusive preserve of a few specialists who are well experienced with protein analysis; since the recent developments in gene technology and immunology the use of sophisticated protein analysis techniques has become widespread.

Protein Sequence Analysis as a Complementary Technique to Nucleotide Sequencing

The recent advances in the purification of proteins and peptides by HPLC and the development of several very sensitive microsequencing techniques have opened new vistas and possibilities in molecular biology and medicine. It has become possible to isolate a gene based on partial protein sequence data by synthesizing oligonucleotide probes for hybridization with gene libraries, isolation of the specific gene, and subsequently to derive the sequence of the entire protein

by recombinant DNA techniques. Other approaches use synthetic peptides manufactured based on partial protein sequences to produce specific antibody, isolate the protein in larger quantity, and conduct functional studies. However, as only a negligible number of the total proteins which can be potentially coded for by eukaryotic genomes are known at this time, there remain major possibilities for research at the protein level and even for the development of a new generation of even more powerful and sensitive protein micro-analytical techniques.

With the development of rapid nucleotide sequencing techniques, however, problems with large-scale isolation of proteins and certain difficulties in the sequence determination are simplified since, by this means, partial amino acid sequences can be extended and peptide fragments easily aligned. Thus, the protein-chemical methods are complemented by nucleotide sequencing once the gene becomes available. Therefore, two chapters of the book are devoted to a description of useful RNA- and DNA-sequencing methods.

However, as has often been emphasized, the direct sequencing of a protein often provides a characterization of the polypeptide structure which is not possible with nucleic acid sequencing alone. In addition, the direct comparison of the nucleotide and protein sequence is especially valuable in the study of organisms where introns may make derivation of the entire protein sequence difficult, based on the nucleotide sequence alone. By N-terminal sequence analysis and determination of the C-terminal amino acids, the putative sequence based on DNA analysis can be confirmed. Furthermore, protein fragmentation can be used to identify functional domains and selective modifications of amino acid residues to locate functionally important regions and active sites of the protein.

Also, by direct amino acid sequencing in contrast to gene sequencing it can be established whether modifications, such as methylations, acetylations, or phosphorylations, occur in a native protein. Proteins frequently contain covalently attached carbohydrate moieties or lipids; the protein itself may be blocked at the N- or C-terminus of the chain, as usually found for short peptide hormones. It is estimated that in eukaryotic cells at least one third of all proteins may be blocked. This number, however, is still uncertain; often inadequate isolation procedures, especially when purifying scarce substance amounts, cause blockage at the N-terminus. Therefore, with well-tested methods on hand, errors at the isolation level of the peptides and proteins can be avoided and the appropriate strategy for sequencing can more easily be selected.

Elucidation of the Protein's Secondary or Tertiary Structure

Information about the secondary or three-dimensional structure of proteins can only be gained if the amino acid sequence is available and the protein isolated in pure form. Establishing the type of secondary structural elements involved in a certain protein, e.g., helical, beta-sheet and beta-turn structures, can be predicted from its amino acid sequence with some certainty (at most with about 60% reliability); however, confirmation of the predicted structure by physicochemical experiments still needs quite large amounts of protein.

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Also, the definitive determination of cysteine or cystine groups in a protein and the elucidation of the sulfur bridges is still laborious and rather complicated. Here, the book provides options for the determination of the cyst(e)ine content of the protein and the location of disulfide linkages using small amounts of polypeptide. Without the direct isolation of the protein under appropriate conditions the S-S bridges cannot be established.

Knowledge of the amino acid sequence is also, of course, a prerequisite for the determination of the protein's three-dimensional structure by X-ray analysis. However, crystallization still needs considerable quantities of protein, often 50 to 100 mg at least. With a substantial reduction in the amounts required for sequence analysis using newer methods more material is now available for these purposes.

After elucidation of the native structure and in combination with gene technology, it is possible to manufacture synthetic proteins, e.g., enzymes with slightly altered properties for special applications or study of functional domains. Further, with the knowledge of the three-dimensional structure, it becomes feasible to synthesize peptides that reproduce the arrangement of the amino acid residues at the protein's surface (topographic antigenic determinants), and to use these peptides for the production of antibodies specific for the native structure of that protein. This is of theoretical and practical importance in immunology and may have medical applications.

Topographical Protein-Chemical Studies on Complexes

Protein-chemical approaches permit investigations on the topography of proteins in organelles or the arrangement of subunits in multi-enzyme complexes. They facilitate the study of DNA- and RNA-protein interactions as well by reaction of the native complexes with bifunctional reagents. After purification of the protein-protein or nucleic acid-protein crosslink, the interacting components can be analyzed and the amino acids and nucleotides involved can be determined.

Strategies for Primary Structural Analysis of Scarce Polypeptide Amounts

Amino acid sequence analysis of increasing numbers of proteins combined with other structural and functional studies illuminates the great diversity and unique properties of proteins. Unlike that of nucleic acids, protein behavior is hard to predict and varies considerably due to the large differences in size, net charge, amino acid composition, solubility, and native secondary and tertiary structure. Hence, generally applicable isolation and characterization schemes cannot be given. This makes protein chemistry rather difficult, and much experience is necessary to manipulate small amounts of protein optimally. On the other hand, this makes dealing with peptides and proteins more challenging compared to other compounds. In order to provide the reader with diverse possible strategies for sequence analysis of structurally disparate polypeptides, one section of the book gives examples of selected strategies for isolation, chemical and enzymatic

fragmentations, amino acid analysis, and microsequencing of such diverse molecules as, for example, membrane proteins, or small-sized peptide hormones. These chapters enable the reader to select the most suitable methods for his own project by reference to methods which were found useful in a similar case.

In addition to the chemical methods for micro-analysis of polypeptides, an outline of the application of mass spectrometry for peptide investigation is included in this book. Recently, mass spectrometry has become very important for the structure analysis of modified peptides and of peptides of unusual structure or composition.

Acknowledgments. We acknowledge the help of Mrs. I. Brauer and Mrs. L. Teppert for co-editing and of Dr. Michael J. Walsh for carefully reading several chapters of the book.

BRIGITTE WITTMANN-LIEBOLD
JOHANN SALNIKOW
VOLKER A. ERDMANN

Chapter 1

Separation and Amino Acid Analysis of Proteins and Peptides for Microsequencing Studies

1.1 Separation of Peptides

ROZA MARIA KAMP[1]

Contents

1	Introduction	8
2	Thin-Layer Fingerprints	9
2.1	First Dimension Electrophoresis	10
2.2	Second Dimension and Detection of Peptides	11
2.3	Elution of Peptides from Thin-Layer Sheet	12
3	High Performance Liquid Chromatography	12
4	Peptide Fragmentation by Enzymatic and Chemical Cleavages in the Micro-Scale	14
4.1	Enzymatic Cleavages	15
4.1.1	Digestion with Trypsin	15
4.1.2	Digestion with Chymotrypsin	15
4.1.3	Digestion with Thermolysin	15
4.1.4	Digestion with *Staphylococcus aureus* Protease	16
4.1.5	Digestion with Pepsin	17
4.1.6	Digestion with Clostripain	17
4.1.7	Digestion with *Armillaria mellea* Protease	17
4.2	Chemical Cleavages	18
4.2.1	Cyanogen Bromide Cleavage	18
4.2.2	Cleavage with BNPS-SKATOLE	18
4.2.3	Partial Acid Hydrolysis	19

1 Introduction

Peptide mapping is a very useful technique for the characterization of proteins. Various rather simple methods can be employed for the separation of peptides, e.g., fingerprinting on thin-layer sheets or a combination of gel filtration or ion exchange chromatography with one- or two-dimensional thin-layer chromatography.

The advantages of these methods are their good resolution and easy production of peptides suitable for direct microsequencing analysis; disadvantages are the low recovery (40 – 70%), depending on the type of peptide and the solvent used for the elution.

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The development of high performance liquid chromatography has revolutionized separation technology of biomolecules. This enabled new purification and fingerprinting techniques for peptide mixtures. The advantages of this method are the very quick separation, high resolution and excellent separation of hydrophobic peptides. The amounts necessary for sequencing peptides separated by HPLC are approximately five times less than that previously necessary by thin-layer fingerprinting; they are 30 times less than that used for a combined open column and thin-layer technique.

The use of these techniques depends on the properties of the peptide mixtures and the equipment of the laboratory. General features and details of sensitive separations of peptides are given for purification by thin-layer fingerprinting or HPLC.

2 Thin-Layer Fingerprints

Peptide mixtures can be separated with good resolution by two-dimensional fingerprinting [1, 2]. The first-dimension peptide separation depends on the peptide's net charges and molecular masses [3]. In the second dimension the peptides are separated by ascending chromatography depending on their individual distribution coefficient. As an example, Fig. 1 shows the fingerprint of tryptic peptides of cytochrome c.

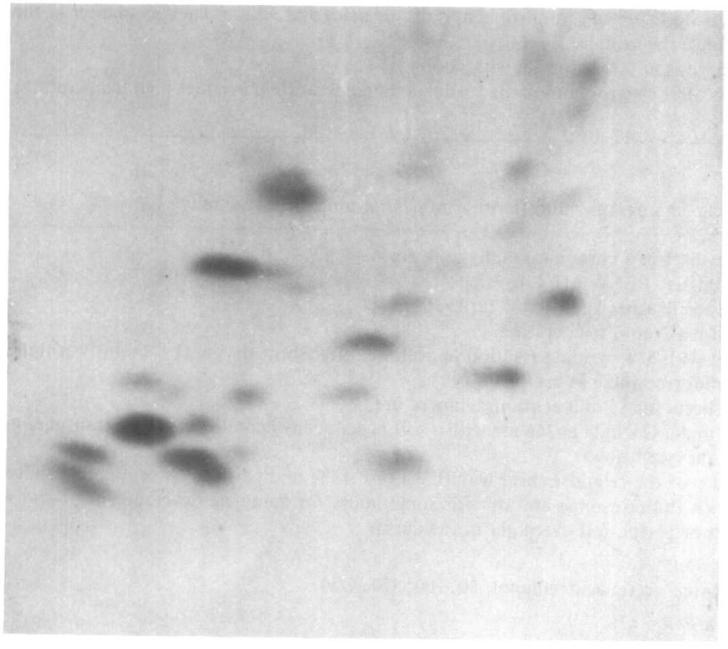

Fig. 1. Tryptic fingerprint of cytochrome c (5 nmol detected with ninhydrin)

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