Chemical Synthesis and Sequencing of Peptides and Proteins

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Developments in Biochemistry Volume 17

CHEMICAL SYNTHESIS AND SEQUENCING OF PEPTIDES AND PROTEINS

Proceedings of the International Conference on the Chemical Synthesis and Sequencing of Peptides and Proteins
held May 8–9, 1980 at the National Institute of Health, Bethesda, Maryland, USA

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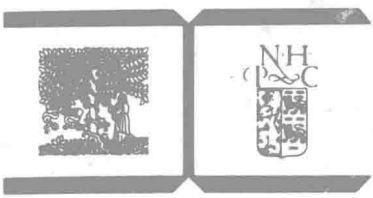

ELSEVIER/NORTH-HOLLAND
NEW YORK • AMSTERDAM • OXFORD

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Published by:

Elsevier North Holland, Inc.
52 Vanderbilt Avenue, New York, New York 10017

Sole distributors outside the United States and Canada:

Elsevier/North-Holland Biomedical Press
335 Jan van Galenstraat, P.O. BOX 211
Amsterdam, The Netherlands

Library of Congress Cataloging in Publication Data
International Conference on the Chemical Synthesis and Sequencing of Peptides and Proteins (1980 : National Institutes of Health)
Chemical synthesis and sequencing of peptides and proteins.

(Developments in biochemistry; v. 17 ISSN 0165-1714)
Includes bibliographies and index.
Contents: The synthesis of peptides / G.T. Young—Chemical synthesis of larger peptides / Ching-I Niu—Chemical synthesis of ribonuclease A with full enzymatic activity / H. Yajima and N. Fujii—[etc.]
1. Peptide synthesis—Congresses. 2. Protein biosynthesis—Congresses. 3. Amino acid sequence—Congresses. I. Liu, Peh-Yung. II. Title. III. Series: Developments in biochemistry; v. 17.
[DNLM: 1. Peptides—Chemical synthesis—Congresses. 2. Proteins—Chemical synthesis—Congresses. 3. Amino acid sequence—Congresses. W1 DE997Vg v.17 / QU 68 I59c 1980]
QP551.I516 1980 574.19'245 81-3307
ISBN 0-444-00623-0 AACR2

Manufactured in the United States of America

Preface

The immediate reason for holding the International Conference on the Chemical Synthesis and Sequencing of Peptides and Proteins was the period of residence at the National Institutes of Health of Dr. Haruaki Yajima as a Fogarty Scholar.

However, if one looks at the program of the Conference, one can see that the topics to be covered reflect a subject of great importance. The determination of the amino-acid sequence of peptides and proteins has become a crucial first step in understanding cellular and organ function. In the last few years, establishing primary structure has been markedly facilitated by new methods of extraordinary sensitivity and, very recently, by analyses at the DNA and mRNA levels.

These advances, in turn, have increased the potential importance of synthetic methods for the production of peptides and proteins, both for the study of structure-function relations and for use as drugs. New methods of solution and solid-phase chemical synthesis have been developed which promise to overcome some of the difficulties of what is a complex chemical problem. On the other hand, biological approaches, using recombinant DNA and cloning methods hold out immense, but only partially tested, potential for production of peptides and proteins.

We hope this conference accomplished two major goals. First, that the formal and informal exchanges among the participants led to new ideas and approaches at the theoretical and at the practical levels. Second, that those other scientists who have need of these techniques for their research will have a better idea of the state of the art.

The Editors of this Conference Proceedings wish to thank Dr. Earl Chamberlayne and his associates of the Fogarty International Center for the skill and efficiency with which the meeting was administered. We are grateful to Drs. C.B. Anfinsen, K.H. Hofmann, S. Moore, and C.H.W. Hius who acted as chairmen of the various sessions.

During the course of planning this conference Dr. William H. Stein died after a long illness. It is obvious that much of the work that was discussed in this Conference was directly descended from his and Stanford Moore's pioneering contributions. Further, many of the participants in this Conference were former students or members of that extraordinary laboratory at the Rockefeller University. For these reasons we have dedicated this Conference to the memory of Dr. William H. Stein.

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Dedication to William H. Stein

Stanford Moore

The Rockefeller University

New York, N.Y. 10021

While this meeting was in the planning stage, William H. Stein died on February 2, 1980. The organizers of this symposium, with the realization that they and most of the scheduled participants had experienced the input of Bill Stein's contributions to the knowledge of protein chemistry, decided that it would be fitting to open these proceedings with a brief essay on his career.

The remarkable achievements being reported at this meeting can perhaps be viewed with fuller perspective if we dwell for a moment on the state of our science in the 1930s, when Bill Stein, after gaining his B.S. degree in chemistry at Harvard, began his researches at Columbia University. He entered the challenging environment of the Department of Biochemistry chaired by Hans Clarke at the College of Physicians and Surgeons. Bill's thesis completed in 1938, concerned amino-acid composition of elastin. In the course of applying some of the gravimetric methods of the time, he used two precipitants which had been developed by Max Bergmann, potassium trioxalatochromiate for glycine and ammonium rhodanilate for proline. A logical outgrowth of Bill Stein's thesis work was his move southward on Manhattan to join the laboratory that Bergmann had organized at The Rockefeller Institute for Medical Research after his arrival in this country in 1934. A photograph of Bergmann (Figure 1) taken in 1939 in his office (now Bruce Merrifield's) on the 4th Floor of what is now Flexner Hall, is a characteristic portrait of the man, with pencil in hand and a twinkle in his eye, as he was about to speak in his soft voice, often with a touch of humor in his phrases. In his researches, he was opening new chapters in the peptide chemistry with which he first became familiar during his apprenticeship days with Emil Fischer in that famous laboratory in Berlin. Today and tomorrow, we are

Figure 1. Max Bergmann in his office at the Rockefeller Institute for Medical Research in 1939.

dealing with the techniques of peptide synthesis and sequence determination. Bergmann contributed to both subjects. To cite only two early examples, he and Leonidas Zervas introduced carbobenzosy chloride as a reagent to yield a reversible blocking group in peptide synthesis, and Bergmann, Kann, and Miekeley showed that a phenylcarbamyldipeptide could be cleaved in acid to a phenylhydantoin and an amino-acid, a reaction that provided part of the chemical heritage upon which Pehr Edman was to draw twenty-five years later.

Bergmann attracted to his laboratory in New York a remarkable group of young investigators dedicated to the study of the chemistry of proteins. Figure 2 is a photograph of Bill taken in 1939 shortly after his arrival at the Rockefeller Institute. At this meeting, we are talking about the sequences of proteins. But in the 1930s it was still an open question as to whether the polypeptide chain of a protein had a specific sequence and adequate methods were lacking for establishing, as a first step, the probable empirical formula of a protein in terms of its constituent amino-acid residues. Bill Stein's first contribution to methodology was his introduction of the solubility product method in an attempt to permit quantitative results to be obtained with reagents which gave sparingly soluble salts of amino-acids. I arrived at the

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Figure 2. William Stein in 1939, shortly after he joined the Bergmann laboratory.

laboratory, via Tennessee and Wisconsin, on the recommendation of my mentor in Madison, Karl Paul Link, who was one of the first chemists whom Max Bergmann visited upon his arrival in the United States from Germany. Link's researches in carbohydrate chemistry drew heavily upon the Emil Fischer tradition in that field. Bill Stein and I, coming to the laboratory with different experiences, happened to make a very suitable team to explore the subject of methods for amino-acid analysis. While Bill was engaged in research on analytical chemistry at Columbia, I was studying with a professor who had just returned from working with Fritz Pregl in Graz. My first instruction from Karl Link was in the minutiae of the microtechniques of Pregl for elemental analysis. Beginning in 1939, Bill and I pooled our efforts for

nearly three years in attempts to extend the scope of the gravimetric determination of amino-acids.

Then came the wartime years during which our teamwork was in abeyance and which saw the untimely death of Max Bergmann in 1944. After the war, the Director of the Rockefeller Institute, Herbert Gasser, had the problem of what do with the two young former assistants of Bergmann. He decided to give Stein and Moore modest space in which to see whether they could develop a research program on their own.

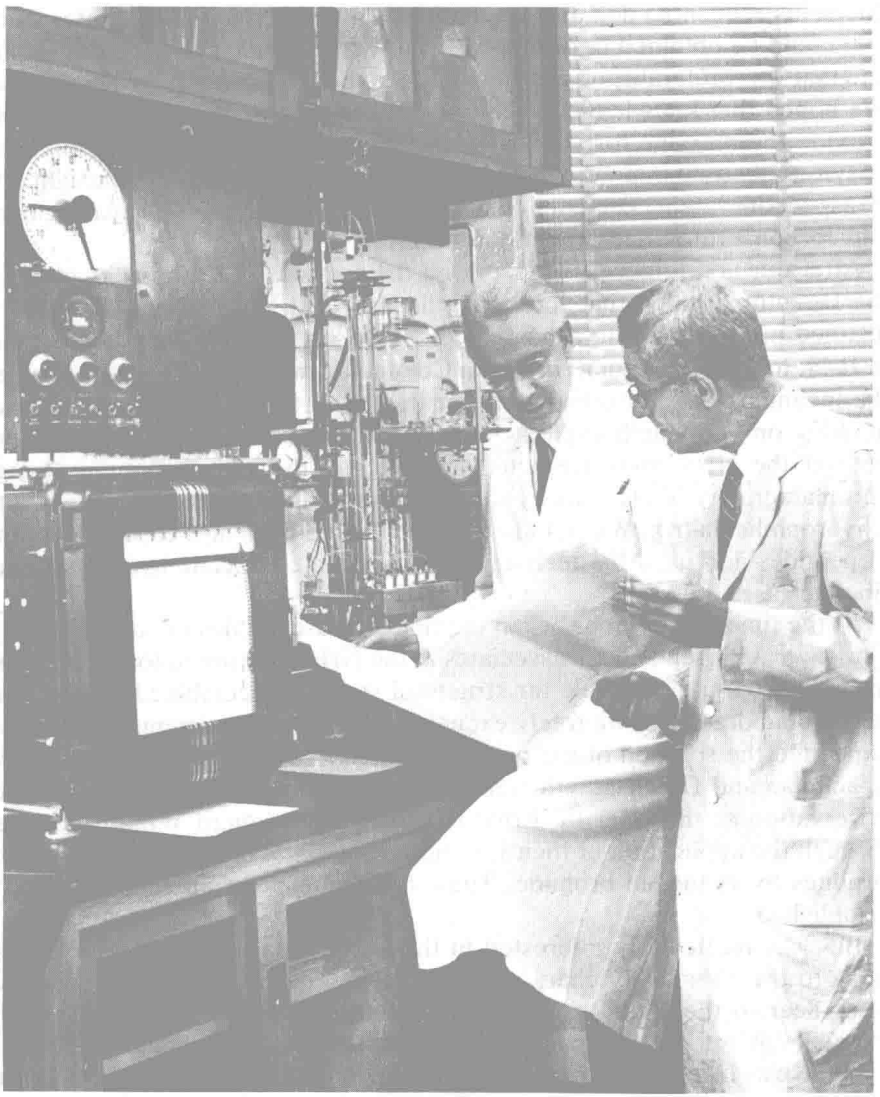
In 1945, it was possible to take a completely new look at the problem of amino-acid analysis. The renaissance in chromatography stimulated by A.J.P. Martin and R.L.M. Synge in England, together with Lyman Craig's development of liquid-liquid countercurrent distribution in his laboratory just down the corridor from ours, brought to the attention of biochemists the potential resolving power of multi-plate separations. We decided to try, as our first attempt in the chromatographic field, to see whether we could render the starch column chromatography of amino-acids quantitative. In order to do this, we concluded that we needed to collect the effluent in a series of small fractions of precise volume. We built a drop-counting fraction collector, with a micro photoelectric eye and an automatic re-set counter, which could be set for a given number of drops. (Before we constructed the instrument, Bill and I tested the column method by using tubes carefully calibrated for 1 ml and we moved a new tube under the column, by hand, as each volume reached the mark. Collection on a time basis was not accurate enough in those days. Sometimes we needed to keep the column running overnight; since I was the bachelor, I got the night shift.)

Then we needed a simple sensitive quantitative method for measuring the concentration of amino-acid in each tube. We turned to the ninhydrin reaction introduced by Siegfried Ruhemann in 1911. The blue color is sensitive to oxidation; in initial attempts the results did not obey Beer's law. We tried carrying out the reaction anaerobically; this improved the yield and the linearity, but was inconvenient. So we undertook to obtain an oxygen-free environment in the solution in an open tube by including a dissolved reducing agent, such as stannous chloride or the reduced form of ninhydrin (hydrindantin). We had to use a water-miscible organic solvent (first methyl Cellosolve, later dimethylsulfoxide) to keep diketohydrindylidene-diketohydrindamine and hydrindantin in solution. We thus attained a reproducible and linear response. After considerable developmental work with carefully purified starch and with alcohol-water solvents, we were able to analyze an acid hydrolysate of serum albumin or β-lactoglobulin quantitatively in two weeks by running three starch columns to resolve all overlaps. The next stage was the development of methodology for the adaptation of the principles of ion-exchange chromatography to the separation of amino-acids and the automation of the resulting process, in cooperation with Darrel Spackman. After more than a decade of developmental work, in the 1960s (Figure

3), we had the pleasure of viewing the results of rapid, automated amino-acid analyses with ion-exchange columns.

During those busy years, Bill and I worked out a system of collaboration, in which we shared the work and the credit, that lasted for a lifetime. Also, during those years, the broad aims born of the Bergmann days were not

Figure 3. William Stein (foreground) and the author at the amino-acid analyzer in the early 1960s.



forgotten. The first user of our starch column procedure was John G. Pierce, then working across the street from us with Vincent du Vigneaud at Cornell University Medical College. They determined the composition of a hydrolysate of purified beef oxytocin as a prelude to the proof of the polypeptide structure of the pituitary hormone by both sequence determination and synthesis. In the 1950s, Frederick Sanger had his classic studies on insulin underway and the polypeptide theory was becoming fact. Quantitative methods of amino-acid analysis seemed to open the field to the study of the structure of larger proteins, and, most importantly, Pehr Edman had introduced his sequential degradation method. Our search for a protein for study that could be obtained in pure form, that was somewhat larger than insulin, and that was preferably an enzyme, led us to bovine pancreatic ribonuclease. We hoped that knowledge of the chemical structure, in this instance could provide the baseline for studies of specific residues which are involved in enzyme-substrate interactions and that thus we could extend the insight into an aspect of protein chemistry on which the studies on catalysis by Bergmann and Joseph Fruton had drawn our attention.

After our first four years of work on the methods of amino-acid analysis, the Director decided that, perhaps, the time had come when we might be justified in being host to a post-docotoral fellow. It was our good fortune, in 1949, to attract Werner Hirs from Columbia University. Werner extended the technique of ion-exchange chromatography to the purification of ribonuclease on a polymethacrylic acid resin; Herbert Sober and Elbert Peterson, here at the NIH, were soon to widen vastly the scope of ion-exchange chromatography of proteins by the key introduction of ion-exchangers with a hydrophilic matrix. Werner also studied the ion-exchange chromatography of peptides and used the technique to fractionate enzymic hydrolysates of ribonuclease.

At the time that Werner began the ribonuclease studies in our laboratory, Christian Anfinsen and his associates at the NIH also turned to ribonuclease as an appropriate molecule for structural study; the combined results from our two laboratories (we freely exchanged data on experiments in progress) expedited the solution of the problem. The final sequence, to which Darrel Spackman and Derek Smyth were contributors, also depended upon a key observation at the NIH by Erhard Gross and Bernhard Witkop obtained through the application of their ingenious method of cleavage at methionine residues by cyanogen bromide. Thus, the sequence of an enzyme became established.

Bill was particularly interested in the application of derivatization reactions to the chemically characterized enzyme in order to identify residues at or near to the active site. Let us take the iodoacetate reaction, as one example. When we started work on ribonuclease, it was known that the enzyme was inactivated by iodoacetic acid. At that time, rapid reaction with iodoacetate was thought of primarily as an indication of SH groups. When

we found that ribonuclease did not have any SH groups, an obvious task was to ascertain what was happening when iodoacetate was added. Through experiments begun by Gerd Gundlach, the reagent was observed to alkylate methionine, histine, or lysine residues in the enzyme. Arthur Crestfield showed that in 30 minutes at pH 5.5 the principal reaction was with the imidazole groups of specific histidine residues. The reagent introduced a carboxymethyl group either on the 1-nitrogen of histidine-119 or on the 3-nitrogen of histidine-12. Through the additional contribution of Robert Heinrikson's data on the effect of carboxymethylation of the ϵ-NH_2-group of lysine-41 on these reactions, it was possible to conclude that in the three-dimensional structure of the enzyme the reactive nitrogens of histidine-12 and histidine-119 were about 5 angstroms apart at the active center of the catalyst and that the ϵ-NH_2-group of lysine-41 was 7-10 angstroms from nitrogen-3 of histidine-12. These three-dimensional predictions, made on chemical grounds, were borne out by subsequent X-ray crystallographic analyses of Frederic Richards and Harold Wyckoff.

Then George Stark wondered whether these two uniquely reactive histidine residues would still be especially reactive to iodoacetate if the molecule were unfolded in 8 M urea. (The answer is that they are not.) He happened to dissolve the protein in 8 M urea at 40° late one afternoon; this was a control without iodoacetate. The next day, when he dialyzed out the urea, the enzyme, which normally snaps back into active confirguration, was diminished in activity. Amino-acid analysis showed a decrease in lysine and an unexpected peak, that turned out to be from homocitrulline. This experiment took our thoughts back to Wöhler in 1828; ammonium cyanate and urea are in equilibrium, and what had happened was that traces of cyanate had carbamylated the ϵ-amino groups of lysine residues to yield homocitrulline. Our publication on this subject simply provided readers with one of the several reminders in the literature that whenever 8 M urea is used, such a reaction is a possibility; it can, fortunately, be avoided by using fresh urea solutions. One of those reminded was Anthony Cerami, then a student at Rockefeller. Some years later when he heard about the possible beneficial effect of administration of urea to patients with sickle cell anemia, he wondered whether cyanate in the urea might be the active reagent. He elicited the cooperation of James Manning, in our laboratory, and out of their work there has grown a decade of research on the effectiveness of the carbamylation of hemoglobin S in converting the molecule to one of almost normal physiological function. In part through serendipity, there has evolved the most effective current therapy for sickle cell anemia—extracorporeal treatment of the blood with cyanate. Bill, throughout his life, in his generous manner, took a special interest in facilitating the careers of scholars whose sojourns in the laboratory made possible the exploration of many facets of the researches. The application of the ion-exchange methods to physiological fluids led Harris Tallan to observations of the presence of 3-methylhistidine

and tyrosine-O-sulfate in human urine and the relatively high concentrations of acetyl aspartic acid in brain; when human brain was analyzed, cystathionine was found to be a major amino-acid. An advantage of the column methods was that sufficient compound could be isolated for full organic characterization. Bill and I emphasized that a chromatogram is no better than the soundness of the reasoning behind the identification of the compounds responsible for the peaks.

The main thrust of the laboratory's effort was toward the purification of enzymes, their chemical characterization, and specific derivatization. The experiments embraced studies on bovine pancreatic deoxyribonuclease, including determination of its sequence, by Paul Price, Tony Hugli, Brian Catley, Johann Salnikow, Ta-hsiu Liao, and Dalton Wang, on streptococcal proteinase (in cooperation with the enzyme's discover, Stuart D. Elliott) by Teh-yung Liu, Brenda Gerwin, William Ferdinand, Michael Lin, and Michael Bustin, on pepsin by T.G. Rajagopalan and T.A.A. Dopheide, on ribonuclease T_1 by Kenji Takahashi, and on carboxypeptidase Y by Rikimaru Hayashi.

This is an international meeting; many of you met Bill during his visits to various parts of the world in his capacities as a conference participant and organizer, visiting professor, trustee, or editor. Many of you remember him as a vigorous participant in the activities of our profession. (One of the advantages of our collaboration was that one of us was prepared to mind the store while the other was on the road.) Even after illness struck him in 1969, in the form of acute polyneuritis, he made the journey to the Nobel ceremonies in 1972. During the last decade of his life, in spite of the frustrations from paralysis, he remained an enthusiastic student of progress in protein chemistry.

One of the pleasures that Bill and I had in the past ten years was seeing ribonuclease biochemistry grow in interest rather than taper off. When we began our work on the enzyme about thirty years ago, we chose the protein as a model molecule for the elucidation of the chemical structure of an enzyme. But we viewed it as a catalyst of rather limited physiological interest; it was recognized as one of the enzymes of the digestive tract. One of the first hints of the broader distribution of enzymes of the same design came from Naples. Enzo Leone and his colleagues have studied the ribonuclease in bovine seminal plasma. It occurs as a dimer the monomeric unit of which is similar to the pancreatic enzyme but is determined by a different gene in the same species.

Then recent studies on cytoplasmic inhibitors of pancreatic-like ribonucleases, first studied in depth by J.S. Roth at the University of Connecticut, have included the isolation of the pure inhibitor from human placenta by Peter Blackburn and Glynn Wilson of our laboratory. Bill lived to see his studies on one ribonuclease provide a basis for studies on a number of them

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