RECOLLECTIONS

The chemical synthesis of proteins

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In 1942 I enrolled in the biochemistry class at UCLA, being taught by Professor Max Dunn, and was introduced into the world of amino acids, peptides, and proteins. The amino acids had been well studied and much was also known about proteins, but no complete amino acid composition was yet known and a sequence of even a small protein was still 9 years away. The idea of a chemical synthesis of a protein was not even discussed. My eventual entry into this field was a gradual process that, in the beginning, was completely unplanned but later became a serious goal. On returning to graduate school 2 years later I served as laboratory assistant in the biochemistry course and began my graduate studies with Dr. Dunn. He was well known for his amino acid syntheses, and his laboratory provided the only source in the world of all of the natural amino acids in highly purified form. They were aptly characterized in a current magazine article as "worth their weight in gold." Dunn (Dunn et al., 1944) had just introduced microbiological methods for the quantitative assay of amino acids, and I joined his group to continue that work. This was the only rapid general method for amino acid analysis and greatly stimulated and accelerated studies on proteins. The chromatographic methods of Moore and Stein had still not been developed.

By 1948 I had extended this microbiological approach to the pyrimidines, for which there was no satisfactory analytical method. I was able to determine that cytosine and uracil were not in equimolar ratio, 0.71, in RNA, and cytosine and thymine were not equimolar, 0.61, in thymus DNA. Unfortunately, I did not have the purine data that would have led to U/A and C/G ratios. The dangers of developing new analytical methods soon became apparent to me when Chargaff's chromatographic techniques did give these ratios

and completely displaced my work. I did, however, manage to isolate for the first time several dinucleotides from partial acid hydrolysates of yeast RNA and to demonstrate that *Leuconostoc mesenteroides* could grow on a complete synthetic medium containing free pyrimidines, but not on mononucleosides or mononucleotides. The dinucleotides, however, were utilized even better than the pyrimidines, provided they contained a nucleoside-3 phosphate esterified to the 5' hydroxyl of a pyrimidine nucleotide or nucleoside.

I moved to New York City in the summer of 1949 and joined the laboratory of D.W. Woolley at The Rockefeller Institute for Medical Research. He had gained considerable recognition for his studies on the vitamins, which were being discovered during his graduate years at the University of Wisconsin, and he was also a pioneer in the field of antimetabolites. Woolley had recently discovered a bacterial growth factor from several protein sources, which he believed to be a peptide. My job was to try to isolate this factor and determine its structure. We succeeded in isolating and purifying two small peptides from partial acid hydrolysates of insulin that had the growth-promoting properties. The first was the pentapeptide Ser-His-Leu-Val-Glu, and the second contained an additional Ala-Leu at the carboxyl terminus. This sequence could be found in Sanger's landmark 1951 structure of insulin (residues 9–15 of the B chain). Other even more active peptides were also present in the insulin digest but were not purified. From this and a collaborative study with Vincent du Vigneaud on his recently synthesized oxytocin and its fragments (Du Vigneaud et al., 1953), it became clear that streptogenin was not a single unique structure like the vitamins, but its growth factor activity was provided by a range of compounds containing either serine or cysteine in peptide linkage.

Our next step was to chemically synthesize the active pentapeptide in order to prove its structure, prepare more material, and provide a route to a range of analogs of the factor (Merrifield and Woolley, 1956). This also led to the design and synthesis of a very effective antagonist. This project was the wedge that led to my long-term interest in synthetic peptides and proteins. In 1956, only a few biologically active peptides were known and only a few of these had been synthesized. But the 1950s was a period of rapid development of synthetic methods both here and in Europe, and they were adequate for the preparation of a pentapeptide. They utilized special reversible amino protecting groups and a variety of carboxyl activation methods. Otherwise, the reactions and methods were similar to the general methods of organic synthesis in which every effort is made to have all reactants fully dissolved in appropriate solvents for all reactions. For larger molecules the solubility can become limiting and the reactions must take place at low

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Bruce Merrifield was born in Fort Worth, Texas, in 1921 and was raised and educated in Southern California. He attended Montebello High School, Pasadena Junior College, and U.C.L.A. with a major in chemistry. After working 1 year he returned to U.C.L.A. and earned a Ph.D. in biochemistry under M.S. Dunn. He and his wife, Libby, moved to New York City in 1949, where he became an Assistant to D.W. Woolley at The Rockefeller Institute for Medical Research. In 1966 he was promoted to full professor, and in 1984 he was appointed John D. Rockefeller Jr. Professor. His main work has been in peptide chemistry and biochemistry. He conceived and developed *solid phase peptide synthesis*. This led to some of the work described in these Recollections and to a number of honorary degrees and awards, including the 1984 Nobel Prize in Chemistry. He serves on the Editorial Boards of the International Journal of Peptide and Protein Research, Peptide Research, Journal of Peptide Science, and Letters in Peptide Science. He is a member of the American Chemical Society, The American Society for Biochemistry and Molecular Biology, The American Peptide Society, and the National Academy of Sciences USA.

concentrations where they are slower and less complete. Isolation, purification, and crystallization of products can often constitute the time-consuming steps of such a synthesis. Using these methods devised by people like Bergmann, Zervas, Wieland, Schwyzer, Anderson, Bodanszky, and others, I was able to assemble and purify my peptide, but only after many months of hard work. It had the same properties as the natural peptide.

At this point it became clear to me that the synthesis of large numbers of analogs of small peptides or the assembly of large peptides or small proteins would be a nearly insurmountable undertaking by a small laboratory like ours and would require the efforts of a large group of expert peptide chemists. For example, there were six authors on the 1953 du Vigneaud synthesis of the octapeptide oxytocin, and subsequently the synthesis of insulin by three independent groups required 6, 10, and 21 people. I felt that a new approach to peptide synthesis was necessary.

One day it occurred to me how we might be able to improve the situation. The idea was to attach the carboxyl group of an amino acid or peptide to an insoluble solid support and to react its amino group with the next amino acid, containing a protected amine function and an activated carboxyl group. This would lengthen the peptide chain by one unit. After deprotection of the amine the process could be repeated over and over until the desired sequence was assembled. There appeared to be three main advantages to such a synthetic scheme: 1, an excess of the incoming amino acid derivative could be used to drive the reaction to completion; 2, the larger peptide component did not have to be soluble as was conventionally necessary, although the support and the peptide would need to be well solvated; 3, since the support would be completely insoluble, the covalently attached peptide would also be insoluble, and the excess soluble reagents, byproducts, and solvents could be removed by simple filtration and washing procedures. There would be no need to isolate and crystallize the intermediate products. I named this process Solid Phase Peptide Synthesis.

Of course, it is one thing to have an idea that you like but quite another to make it work. And I spent a long, long time trying to make this one work. The difficulty was to get all the variables worked out at the same time because they were interdependent. Eventually, after 3 years of sometimes exciting and sometimes frustrating effort the synthesis of a small tetrapeptide succeeded and the work was presented in 1962 and published in 1963 (Merrifield, 1963). I was much encouraged to push ahead with the development and application of the new method. Rather quickly I was able to synthesize the nonapeptide bradykinin, in good yield and purity, and it had full biological activity. My first graduate student, Garland Marshall, was able to synthesize the octapeptide angiotensin. At this stage of development the support was a copolymer of styrene and 1% divinylbenzene crosslinker. The first amino acid was temporarily protected with an *N*^α-*tert*butyloxycarbonyl group and was attached to the resin as a benzyl ester. Activation of subsequent amino acids was with dicyclohexylcarbodiimide, and the solvent was dichloromethane.

However, my troubles were not over. Many of the most established peptide chemists did not believe such a method could work or give products that would meet their high standards. Others were more broad minded and actually tested the method. Of these, some were successful and some were not and quickly signed off. But the successful ones became very enthusiastic and began to make sizeable numbers of very useful peptides that met their needs. A number of well-known non-peptide chemists also indicated their approval of this new general method for synthesis of organic oligomers. Sir

Robert Robinson was one who liked the idea. Even without the outside reactions it was clear to me that this was only a beginning and that I would have to study all the variables in much more detail to develop a good general method. This became a long-term activity in my laboratory and eventually in a number of other laboratories. The details of the early developmental work and subsequent improvements have been reviewed a number of times (Barany and Merrifield, 1979). We examined other solid supports and their physical and chemical properties. We studied a variety of protecting groups and a number of activating reagents and solvents, and different reaction conditions. As various side reactions were observed or reported by other laboratories, we tried to understand them and to find ways to overcome them. So solid phase peptide synthesis became better understood and slowly improved. I could now begin to think of larger peptides and even dream about a small synthetic protein.

From the beginning of the solid phase idea it seemed reasonable that the process would lend itself to automation because the solid support allowed all reactions to be conducted in a single reaction vessel, and the physical manipulations consisted of simply adding and removing soluble reagents and byproducts while the growing peptide chain remained in the vessel. My first attempts were by a flow system in a packed column, but technical problems led to the use of a batch system in which the resin was suspended in solvent in a closed vessel and after reaction the solvent was removed through a glass filter under vacuum.

To mechanize this process I enlisted the collaboration of Dr. John Stewart, who also worked in the Woolley laboratory. Together, in 1965, we constructed an instrument that would carry out all steps required for extending the peptide chain by six residues in a 24-h period (Merrifield et al., 1966). Reservoirs were then filled with additional amino acid derivatives and the synthesis was continued. The process was controlled by an electromechanical stepping drum programmer. Currently, this has been replaced by microprocessors and computers in several commercial synthesizers. Effective flow systems have also been developed.

With the new machine I could realistically think about the synthesis of a protein. Emil Fischer (1906) was the first to seriously consider the synthesis of a molecule that might have some of the properties of a protein. This was in spite of his very limited synthetic methodology and the fact that not even the composition, let alone the sequence of any protein, was known. Many believed proteins were not long-chain polymers, but were heterogeneous colloids composed of small molecules. I like very much his now famous quotation: "Whereas cautious professional colleagues fear that rational study of this class of compounds, because of their complicated structure and their highly inconvenient physical characteristics, would today still uncover insurmountable difficulties, other optimistically endowed observers, among which I would count myself, are inclined to the view that an attempt should at least be made to besiege this virgin fortress with all the expedients of the present; because only through this hazardous affair can the limitations of the ability of our methods be ascertained."

He accomplished the synthesis of an 18-residue peptide composed of D,L-leucine and glycine, which did have some properties of a protein. Fischer himself believed proteins were approximately 30 to 40 residues in length and that longer chains would be unstable. Following Fischer's work and that of his former student Abderhalden, who made a 19-residue peptide, no proteins were synthesized until the independent insulin work from the laboratories of Katsoyannis, Zahn, and the Shanghai Institutes of Biochem-

istry and Organic Chemistry during the 1963–1965 period. These all involved fragment synthesis of the 21- and 30-residue A and B chains and their assembly into the 51-residue insulin by careful oxidation of the six cysteine residues. The last step was the difficult one and usually went in low yields.

Now was the time, I believed, to test our methods on a long protein chain to determine whether they could be used to study these very inaccessible molecules. I selected ribonuclease A as a target molecule because its 124-amino acid sequence had been determined by Hirs, Moore, and Stein and its crystal structure was known from the work of Kartha, Bello, and Harker, and that of Ribonuclease S by the Wyckoff and Richards group. Equally importantly, Anfinsen and his collaborators had shown that the protein could be reduced and denatured and reoxidized and renatured to give full recovery of activity.

Bernd Gutte joined me in 1967, and began work on this project. He used the machine and the methods described for bradykinin, and by the spring of 1968 had obtained RNase-active material of 13% specific activity. It was purified and characterized by a variety of methods and published as a note in *The Journal of the American Chemical Society* in January of 1969 (Gutte and Merrifield, 1969). After further purification and characterization a detailed full paper was published in the *Journal of Biological Chemistry* (Gutte and Merrifield, 1971). The protein gave good analytical data for homogeneity, composition, and sequence. The enzyme was active against large RNA and small cyclic nucleotide substrates, demonstrating that it catalyzed both the transphosphorylation and hydrolysis steps. It showed high specific activity (80%) and correct substrate specificity for pyrimidines, not purines, and ribose, not deoxyribose, and the protein was neutralized by an antibody to natural RNase A (Fig. 1).

The JACS communication was accompanied by reports of the synthesis of RNase S by the Merck group headed by Robert Denkewalter and Ralph Hirschmann using a fragment approach in solution together with their new N-carboxyanhydride method. They achieved the synthesis of S-protein (RNase 21–124), and then the non-covalent addition of S-peptide (1–20) constituted a synthesis of RNase S (Hirschmann et al., 1969). It was enzymatically active.

Our RNase A synthesis allowed us to draw the following conclusions: 1. It is possible to begin with free amino acids and to

assemble them into a real protein with true enzymatic activity. 2. The primary structure of a protein can determine its tertiary structure. This conclusion, based on a synthetic protein, was a new and independent confirmation of Anfinsen's findings based on the natural protein. Gutte went on to make a series of analogs of the enzyme by expanding on Richards' finding that upon mixing the N-terminal S-peptide (RNase 1–20) and S-protein (RNase 21–124) derived by enzymatic cleavage of native RNase, high RNase activity could be regenerated. We synthesized the C-terminal tetradecapeptide (RNase 111–124) and showed that it would regenerate activity when noncovalently mixed with shortened RNase 1–118. This semisynthesis approach led to a variety of synthetic replacement analogs of the C-terminal peptide and provided considerable evidence for the roles of several residues at that end of RNase in the activity of the enzyme. Previously, both Hofmann's group and Scoffone's group had done similar studies on synthetic S-peptide and natural S-protein. Bob Hodges then showed that a synthetic recombinant could be designed that was more active than native RNase and another was made that had selective substrate specificity. Thus, the Tyr¹²⁰ protein was 2.1 times more active than the native enzyme containing Phe¹²⁰, and the Ala¹²¹ derivative was 19 times as active toward cytidine cyclic phosphate, as it was against uridine cyclic phosphate (Hodges and Merrifield, 1975). This was a first step towards the design of a pyrimidine-selective ribonuclease. We were especially intrigued by a demonstration by Michael Lin et al. (1970) that, for the first time, a non-covalently bound three-component protein system could be constructed that displayed enzymatic activity. Thus, when RNase 1–20 containing His¹², RNase 111–124 containing His¹¹⁹, and RNase 21–118 containing Lys⁴¹ were mixed, good activity was generated. In this case, each of the three known catalytic residues was present in a separate peptide and they could properly reassemble into a structure that could function as an enzyme.

Although I have concentrated on our own work up to here, it must be emphasized that several other laboratories had also been interested in synthesizing proteins. I have already mentioned the insulin syntheses by the classical fragment condensation methods. Assembly of larger proteins by this method were, of course, much more difficult. Hofmann attempted the assembly of RNase T₁ (104 residues) from 14 small synthetic fragments, but the total synthesis failed. Kenner's group made a major effort to synthesize an analog of lysozyme (129 residues) but the formation of the four disulfide bonds proved insurmountable. The successful fragment synthesis by the Merck group has already been mentioned. Ten years later, Fujii and Yajima completed an elegant fragment synthesis of RNase A. They wisely preferred to couple small fragments to the large growing peptide chain and found fewer solubility problems and higher yields. After physical purification steps their specific activity was virtually the same (13%) as we had found in our stepwise solid phase synthesis, but they were able to raise it to ~100% by affinity purification. The fragment syntheses were traditionally plagued by problems of insolubility of the large intermediates and of racemization when the activation was not at a glycine or proline. However, in recent years most of these difficulties have been overcome and in the hands of experts amazingly good syntheses of peptides in the 50- to 100-residue range are being achieved.

Similarly, other syntheses of proteins by stepwise solid phase methods were being reported in the late 60s and early 70s. These included ferridoxin (55 res.) trypsin inhibitor (58 res.), cobrotoxin (62 res.), acyl carrier protein (1–74), RNase T₁, lysozyme, *Staphylococcus nuclease* semisynthesis (1–49 + 50–149), and a growth

Fig. 1. Bernd Gutte (left) and Bruce Merrifield in front of the machine used to synthesize RNase A. Reproduced from *Chemical and Engineering News*, 1969, vol 47, pp 62–63, with permission of the American Chemical Society.

hormone analog (188 residues). These proteins varied a great deal in their yield and purity, and this period must be considered an exploratory time where methodology was being tested.

The solid phase synthesis of proteins continues at a good rate and products of excellent quality are being reported. At least one synthetic protein was obtained in crystalline form suitable for X-ray diffraction studies. Some of the more recent syntheses include: S-chorionic gonadotropin (74-residues), ubiquitin (76), interleukin-8 (77), Tat protein (86), preprogonadotropin-releasing hormone (92), Gro ES chaperonin (97), HIV-1 protease (99), prothymosin a (109), leukemia virus protease (126), cardiodilatin (126), colony-stimulating factor (127), HIV-1 nucleoprotein p24 (134), interleukin 3 (140), and interferon (166).

Most of these proteins have been prepared by the discontinuous batch procedure, but some have been by continuous flow methods. Although the basic principles are the same, we continue to see changes in all steps of solid phase synthesis, and the resulting improvements are, of course, what we are all seeking.

This more or less brings me to the end of my "recollections" period. But this is still a viable field being developed not only in my laboratory but especially in a number of other laboratories. My tie to much of the new work is through the solid phase method and, therefore, I feel justified in commenting on some of the important new developments.

Nowadays, the question often asked is "why did you want to chemically synthesize peptides or proteins?" During the period covered here the goals were to develop this field of synthetic organic chemistry and to use the new techniques to prepare and study a wide range of biologically active compounds. This could not be done easily by other methods, and a great deal of interesting and valuable information was obtained, leading to more active agonists, to effective antagonists, and to a much better understanding of some of the mechanisms of action of these peptides. Even with the sophisticated DNA technology now available and the ability to make point mutations leading to the expression of a range of modified proteins, there are still valid reasons why chemically synthesized peptides and proteins are important. These include ease and speed of synthesis, high yields, the introduction of unnatural amino acids and other components, preparation of very large numbers of peptides and peptide mimetics needed for screening and drug design, and introduction of specific labels. I believe, therefore, that the two fields are highly complementary.

New approaches for efficient and selective fragment synthesis of proteins are currently being developed in several laboratories. They depend on a specific bimolecular chemical reaction to form the peptide bond between two purified fragments, or the formation of an intermediate by a selective capture method that brings the carbonyl of one peptide and the amine of the other peptide very close together for an intramolecular reaction, which occurs at very high effective concentration in the molar range. These are generally called ligation methods. Efforts are also directed toward procedures that require minimal or no side-chain protection so that aqueous coupling systems can be used and denaturing or possible destructive deprotection steps in organic solvents can be avoided. These methods look promising for preparations of proteins in the 100- to 200-residue range. Daniel Kemp is considered to be the originator of this approach, and now my former colleagues James Tam and Stephen Kent are two of the leaders in the field. The peptide fragments are usually prepared by solid phase methods, and after deprotection and purification they are condensed in aqueous solution by the above methods.

I think the hottest new area in which solid phase peptide synthesis plays a key role concerns the rapid simultaneous synthesis of multiple peptides. This was initiated by Mario Geysen and his colleagues in a search for antigenic epitopes of proteins and was soon followed by the tea bag method of Richard Houghten in which the original single glass reaction vessel was replaced by many small bags of polypropylene mesh. More recently, combinatorial libraries containing m^n peptides are being used by many laboratories and especially by pharmaceutical companies looking for new drugs. If all possible peptides of n residues are made from m different amino acid monomers, the numbers of products becomes very large. For example, all pentamers made from 18 amino acids gives 1,889,568 peptides! These can be screened in various ways and the most active products eventually identified. Houghten uses his tea bag technology and iterative methods while others use the divide-and-recombine method of Furka et al. (1991), together with the one-bead one-peptide insight of Kit Lam et al. (1991). In the latter approach, every resin bead will be associated with a single peptide sequence and all peptides will be represented in the population of beads. These are all based on a resin-bound synthesis, but phage libraries have also been prepared. These are very powerful methods with enormous potential, especially when unnatural peptide derivatives or nonpeptide monomers are used.

For several years attempts have been made to design and synthesize proteins *de novo*, unrelated to any natural molecules, that would adopt predictable conformations and exhibit specified chemical or biological properties. For example, Gutte designed a 34-residue peptide that would form a $\beta\beta\alpha$ secondary structure and bind an oligo nucleotide (GAA), and another that would bind DDT. Bruce Erickson and his group have worked for a long time, beginning when he was in our laboratories at The Rockefeller University (Erickson et al., 1986) and continuing at the University of North Carolina, on the *de novo* synthesis of a 68-residue chain that would fold into a predicted β -barrel structure, and which would eventually be extended to contain specific catalytic sites. Perhaps the most successful efforts along these lines have been by Manfred Mutter using a scaffold support to align, for example, a four-helix peptide bundle that would react with antibodies to the major histocompatibility complex. William De Grado has made a linear 73-residue peptide, and further engineering produced a zinc-binding protein. His hope is to synthesize an enzyme. As the understanding of the forces that control protein conformation becomes better, we can expect significant new developments in this field.

A new and creative use of peptides was recently introduced by Nielsen, Egholm, Berg, and Buehler (1991), in which they have designed and synthesized nucleic acid derivatives named protein nucleic acids (PNAs). In these, the sugar phosphate backbone was replaced by a branched amino acid derivative still containing the purine and pyrimidine side chains of approximately the same spacing as in DNA. The monomer units for each base are assembled on a solid support just as natural amino acids would be. The PNAs lack the strong negative phosphate charges and bind so tightly that they can displace one DNA chain from its nucleic acid duplex. Biotin-labeled PNAs have been used by Vincent Allfrey and his group (Boffa et al., 1995) to specifically isolate an actively transcribing gene from a cancer cell.

I wonder what Emil Fischer would say about all these exciting developments in the field he created. I do not think he would be surprised, but would be enormously pleased.

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