Journal of Peptide Science
J. Peptide Sci. **6**: 1–10 (2000)

Review

My Journey from Wool Research to Insulin[1]

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Received 16 September 1999
Accepted 20 September 1999

Abstract: This paper is an autobiographical study of the author's early work on the chemical cross-linking of proteins as well as on oligomer and peptide synthesis from 1949 in Heidelberg until the synthesis of insulin in 1963 in Aachen[2]. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: chemical cross-linking; difluorodinitrobenzene; difluorodinitrodiphenylsulfone; nitrophenylesters of dicarboxylic acids; oligomers of aminocaproic acid; fibroin peptides; insulin synthesis

INTRODUCTION

The elucidation of the covalent structure of insulin by Sanger [1] in Cambridge formed the basis for all other work on this molecule. The chemical synthesis, the discovery of proinsulin by Steiner [2] in Chicago and Chance [3] in Indianapolis, the solving of the three-dimensional structure of 2-zinc porcine insulin by Hodgkin and her associates [4], and finally the biosynthesis of human insulin via genetic engineering [5] were further milestones.

In spite of extensive coverage of the history of the various insulin syntheses, people still wonder why the first chemical synthesis [6] was achieved in the German Wool Textile Research Institute in Aachen.

Here I describe my journey from the synthesis of cross-linking agents for wool and silk and from the synthesis of oligomers and peptides to the cross-linking and synthesis of insulin.

MY JOURNEY TO INSULIN STARTED OFF WITH CROSS-LINKING WOOL

The changeover in my professional activities from Textile to Organic Chemistry in Heidelberg in Karl Freudenberg's Institute for Chemistry let me dive into an atmosphere characterized by the many-sided research activities of the staff members (Table 1).

First I worked on topics which I was familiar with from my wool research work in Egon Elöd's Institute for Textile Chemistry. Cross-linking reactions were studied under the aspect of practical application in the repair of wool and silk damage caused by wet processing such as bleaching or dyeing. Other uses included the protection of wool from damage by acids, alkali and micro-organisms. I could confirm the cross-linking efficiency of the bischloromethylether of butan-1,4-diol [8]. I was, however, unable to deliver unambiguous chemical evidence for the location of the reagent or of formaldehyde within the wool proteins [9]. It was this disappointment that made me interested in other cross-linking agents that could react with wool and silk while forming stable covalent bonds. Sanger's [10] reagent 1-fluoro-2,4-dinitrobenzene (FDNB) pointed the way to its bifunctional analog 1,5-difluoro-2,4-dinitrobenzene (FFDNB), which I got to know purely by chance during a discussion with Rudolf Löwenfeld, a co-worker of Friedrich Weygand. FFDNB was reacted with methylamine, arabinamine and galactamine [11] (Figure 1).

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[1] Lecture given at the 4th Deutsches Peptidsymposium, University of Leipzig, 21–24 March 1999 (Chairman Hans-Dieter Jakubke).

[2] Editor's note: Professor Zahn was born in 1916 in Erlangen and studied chemistry at, and graduated from, the University of Technology in Karlsruhe (Dr. Ing. in 1940). From 1940 to 1949 he was assistant to Professor Elöd at the Institute for Textile Chemistry in Karlsruhe and later in Badenweiler, and from 1949 to 1957 he was assistant to Professor Freudenberg at the Institute for Chemistry of the University of Heidelberg. From 1952 to 1985 he was Director of the German Wool Research Institute, and in 1960 was appointed full Professor at the University of Technology in Aachen.

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Table 1 List of the Academic Staff at the Institute for Chemistry in Heidelberg 1949–1958 [7]

Inorganic chemistry	Organic chemistry	
Rolf Appel	Friedrich Cramer	Otto Th. Schmidt
Margot Becke-Goehring	Hans von Dietrich	Walter Stumpf
Harry Hahn	Karl Freudenberg (Director until 1956)	Hans-Joachim Teuber
Robert Juza	Walter Lwoswski	Klaus Weinges
Reinhard Nast	Walter Mayer	Friedrich Weygand
	Hans Plieninger	Georg Wittig (Director since 1956)
		Helmut Zahn

To me it was obvious that FFDNB is a bifunctional Sanger reagent and should be a new and promising cross-linking agent. Wool fibres that were treated with FFDNB had indeed a lower solubility than untreated fibres in a variety of solvents. They also supercontracted to a lesser extent. All of these changes in properties indicated the introduction of cross-links [12,13].

Direct proof of cross-linking was obtained when we identified three pairs of cross-linked amino acids in the acid hydrolysate of the treated wool (Figure 2). Monofunctional reaction of FFDNB with lysine and

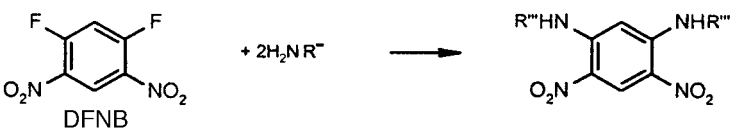

Figure 1 Condensation of 1,3-difluoro-4,6-dinitrobenzene with amino compounds [11].

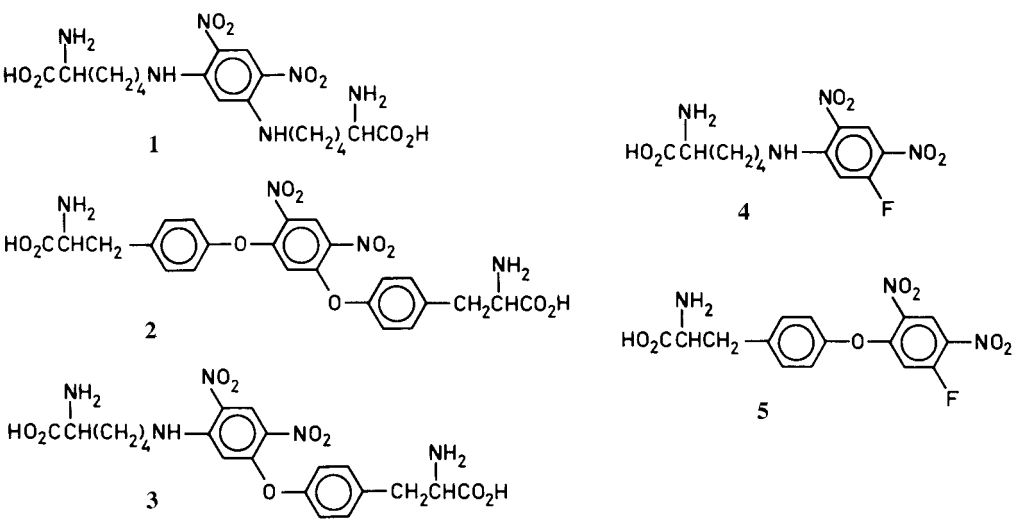

Figure 2 Products of the reaction of FFDNB with wool: (**1**) *N,N′*-(2,4-dinitro-1,5-phenylene)dilysine; (**2**) *O,O′*-(2,4-dinitro-1,5-phenylene)dityrosine; (**3**) *N,O*-(2,4-dinitro-1,5-phenylene)lysine,tyrosine; (**4**) *O*-(2,4-dinitro-1-fluoro-5-phenyl)tyrosine; (**5**) *N*-(2,4-dinitro-1-fluoro-5-phenyl)lysine.

tyrosine residues gave two further reaction products [14] (Figure 2).

All five of these compounds were separated by paper chromatography and identified by comparison with authentic samples.

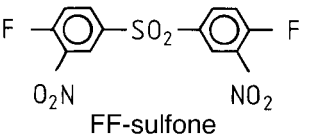

Figure 4 4,4′-difluoro-3,3′-dinitrodiphenylsulfone

REACTION OF DINITRODIFLUOROBENZENE WITH FIBROIN

The main reaction of FFDNB with fibroin was the cross-linking of the tyrosyl side chains. Dinitrophenylene–dityrosine **2** was isolated from the hydrolysate of the cross-linked fibre preparation in high yield [15] (Figure 3).

These experiments indicated a preferred position of tyrosyl residues in certain domains of the fibroin molecule.

The maximum bridge span of FFDNB is 0.6 nm. In an attempt to determine the distances between adjacent peptide chains in the noncrystalline domains of fibroin, a series of bifunctional fluorine-containing reagents with molecular lengths of 0.3–1.4 nm were synthesized by Zahn and Zuber [16].

After reacting fibroin with such compounds we were able to establish that a bifunctional reaction had taken place.

Maximum cross-linking occurred in fibroin when the reactive molecule was about 1.0 nm in length. Thus, with 4,4′-difluoro-3,3′dinitrodiphenylsulfone (Figure 4), a 100% bifunctional reaction with tyrosine residues was achieved.

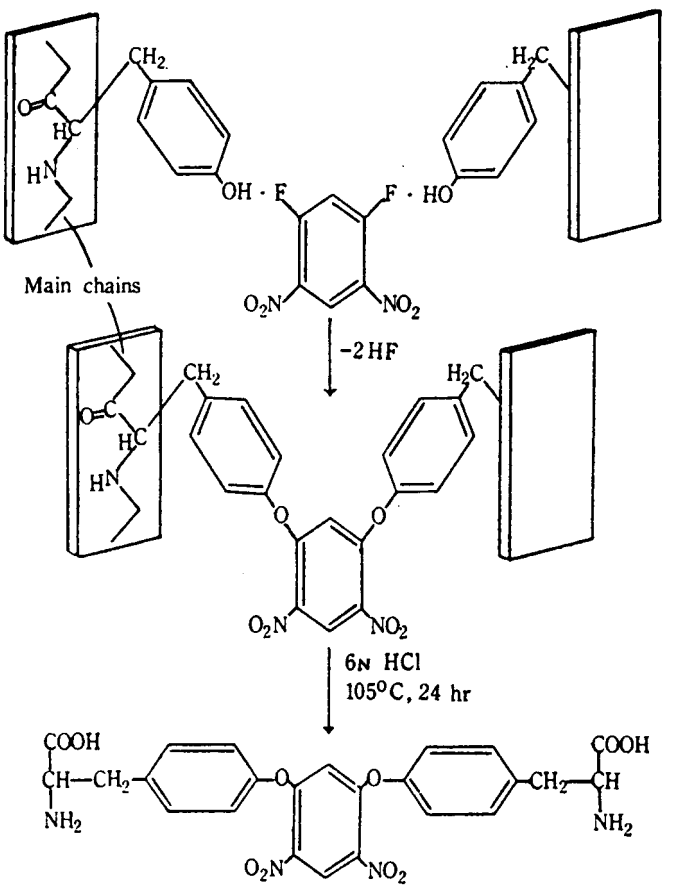

Figure 3 Schematic representation of the formation of cross-links between tyrosyl residues in silk fibroin by treatment with FFDNB. Isolation of *O*,*O*′-dinitrophenylene-dityrosine.

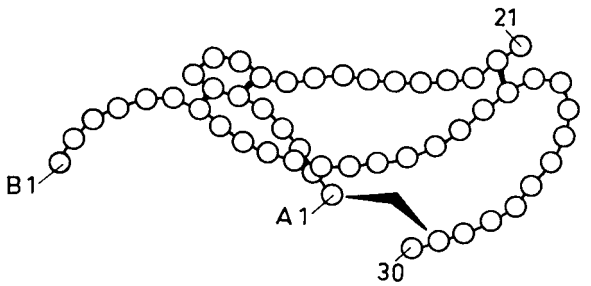

Figure 5 Schematic representation of the dinitrophenylene bridge between the amino groups of A1-glycine and B29-lysine in insulin [20].

The introduction of chemically stable synthetic cross-links into fibroin gave valuable information concerning the molecular structure of the noncrystalline domains of silk fibroin. As far as collagen is concerned, the unexpected proximities of some hydroxylysine residues were established by Wegerle [17] with 4,4′-difluoro-3,3′-dinitrodiphenylsulfone (FF-sulfone) as a cross-linking reagent.

CROSS-LINKING THE AMINO GROUPS OF A1-GLYCINE AND B29-LYSINE IN INSULIN

From 1949 to 1953 only the reactions of bifunctional and heterobifunctional reagents with fibrous proteins were studied. It was obvious for us to study the suitability of our reagents for determining the distances between nucleophilic groups in proteins in solution.

Insulin was the ideal protein. Meienhofer began his diploma thesis in July 1953 [18]. In those days the covalent structure of the A and B chains [19,20] was known, but neither the position of the disulfide bonds nor the molecular weight had been established unambiguously. Meienhofer synthesized all the amino acid derivatives of FFDNB and FF-sulfone which were expected from reactions with insulin and were not yet available. One series of experiments on cross-linking insulin with FFDNB was carried out at low insulin concentrations in alkaline solutions and in dimethylformamide. The intramolecular reaction resulted in the formation of a cross-link between the amino groups of A1-glycine and B29-lysine far apart from each other. Our conclusion [19] was, that in the tertiary structure

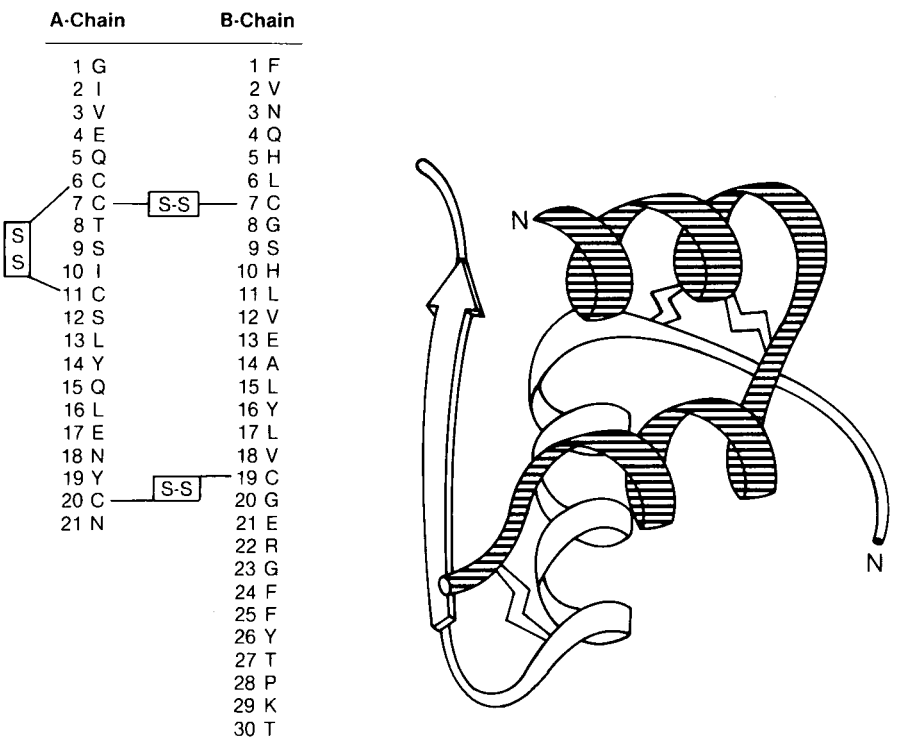

Figure 6 The amino acid sequence of human insulin and a ribbon drawing of the monomeric structure (the A chain is striped) [21].

the *C*-terminal end of the B chain is located above the *N*-terminal end (Figure 5).

Evidence relating the conformation of monomeric insulin in solution to that found in the crystal was not available before 1969 [4], when Hodgkin solved the crystal structure of insulin.

Let me quote from her publication:

> "In the central part of the B chain, there are three turns of α-helix, slightly opened out at each end. Both the interchain disulfide bonds, B7–A7 and B19–A20, are at the ends of the helix which provides a rigid backbone. B1–B6 and B21–B30 are largely extended and loosely packed around the A chain. The two residues, A4 glutamic acid and B29 lysine, appear to be in contact as expected by Zahn (private communication)".

A comparison with the Sanger structure illustrates that the proximity of A1-Gly and B29-Lys in crystalline insulin could not be expected by looking at the plot of the primary structure (Figure 6).

Cross-linking A1-glycine with B29-lysine later became a successful field of research for Brandenburg [22]. FFDNB was no longer applied and replaced by the 4-nitrophenylesters of dicarboxylic acids. They were first used for cross-linking wool, silk and collagen [23–25]. Bis-benzyloxycarbonylcystine-bis-*o*-nitrophenylester was incorporated into collagen as a synthetic cystine bridge with a cleavable disulfide bond [26]. With the discovery of proinsulin [2,3] the synthesis of 'miniproinsulins' by cross-linking A1-glycine with B29-lysine with active esters of dicarboxylic acids led to interesting results on structure–function relationships [27] (Figure 7).

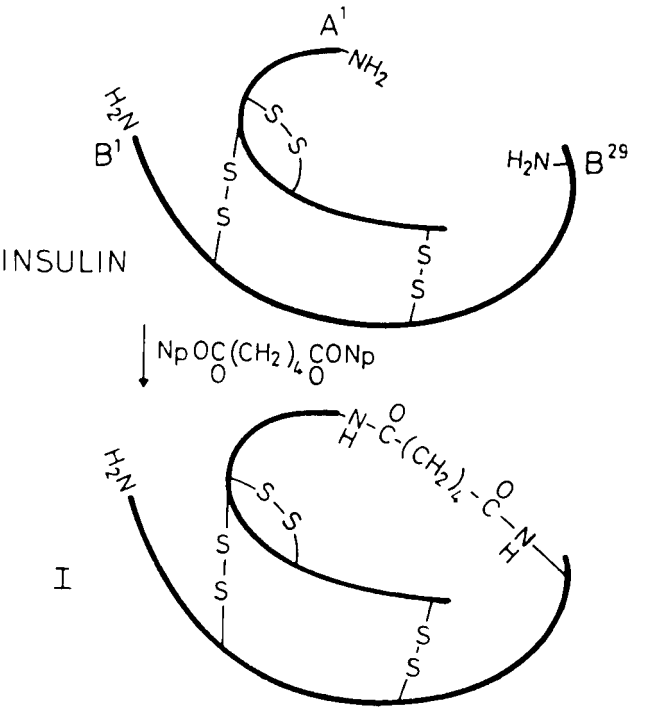

Figure 7 Plot of the reaction of adipic acid bis-*p*-nitrophenylester with the amino groups of A1-glycine and B29-lysine in insulin to adipoyl-insulin [22].

I measured the lamellar stacking periodicity (LSP) in nylon 6 lamellar crystals from oligamides of the type $C_6H_5CH_2OOC–(NH(CH_2)_5CO)_nOH$ within the range 2–12 and found a linear correlation with increasing chain length up to the octamer (length ~ 8 nm), beyond which the value of LSP remained constant [31] (Figure 8).

SYNTHESIS OF OLIGOMERS AND PEPTIDES

My work on the synthesis of peptides in Heidelberg was started to find out the onset of X-ray patterns of the corresponding polymer. The first example was nylon 6.6. It was shown by Zahn and Rathgeber [28] that already the low molecular 'nylon peptide' adipoyl-bis-hexamethylendiamine $H_2N(CH_2)_6NH–CO(CH_2)_4CO–NH(CH_2)_6–NH_2$ delivers the same X-ray pattern as nylon powder. The field of oligomer research became the main area of my activities in Heidelberg and later in Aachen [29,30]. Not only were typical X-ray patterns of the crystalline domains found, but also low angle X-ray patterns of chain-folded lamellae in oligomers above a certain chain length.

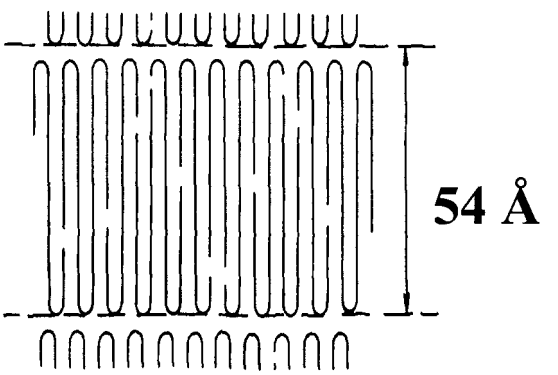

Figure 8 Plot of an assumed conformation of benzyloxycarbonyl-(ε-amino-caproyl)$_9$OH in a chain folded state. X-ray long spacing 5.4 nm found, 8.9 calculated for the extended chain [31].

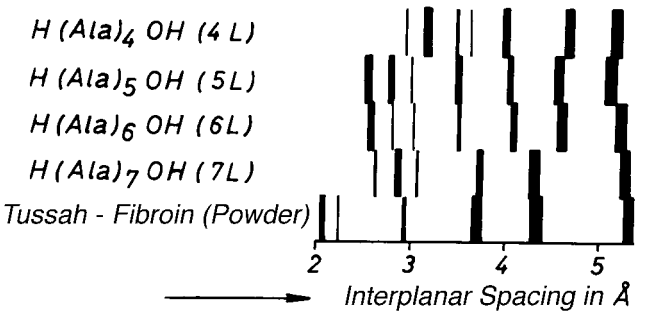

Figure 9 Plot of the X-ray pattern of oligomers of alanine and Tussah-fibroin powder [36].

Zahn and Pieper [32] established that above the octamer the LSP values were dependent on solvent and crystallization conditions, while for the octamer and lower oligomers the LSP values were solvent-independent.

The same question has been the motivation for our work on synthetic fibroin peptides. We wanted to know if the β-pleated structure of silk fibroin would occur in peptides having sequences of the crystalline domains.

These consist, to a great extent, of hexapeptide repeat units of the sequence Ser-Gly-Ala-Gly-Ala-Gly [33]. Zahn and Schnabel [34] succeeded in synthesizing this peptide and we could show that the substance came up with X-ray reflections mostly identical with those of the isolated crystalline domain of silk fibroin.

The X-ray pattern of Tussah-fibroin and β-polyalanine are similar [35]. We were interested to find out the onset of the Tussah-fibroin-X-ray pattern in a series of alanine peptides.

Zahn and Meißner [36] synthesized the whole series of oligoalanine up to the heptapeptide. The microcrystalline powder of the heptapeptide gave the typical Tussah-fibroin X-ray pattern.

This was a strong indication of an extended β-chain structure of heptaalanine (Figure 9).

As shown earlier (page x) the high yields of cross-linked tyrosine residues have been an argument for the assumption that the tyrosine residues are located in the noncrystalline domains of fibroin. We tried to identify some tyrosine peptides isolated from fibroin by synthesis of the expected amino acid sequences, but failed. It was therefore no wonder that we directed our interest in tyrosine peptides to the insulin molecule, which contains four tyrosine residues. With the data on a possible role of tyrosine residues for insulin activity by Fraenkel-Conrat in mind [37] Messerknecht synthesized our first insulin peptide, Try-Leu-Val [38]. The tyrosine peptides Gly-Phe-Phe-Tyr and Ala-Leu-Tyr-Leu-Val were synthesized by Zahn and La France [39,40].

SYNTHESIS OF INSULIN

In the spring of 1957 I moved to Aachen. The inauguration ceremony of the German Wool Research Institute was held on the 10th of May. The President encouraged me to continue organic chemistry work as a means of elucidating the fundamentals of wool processing and finishing. The synthesis of oligomers and insulin B chain peptides was resumed. A great step forward for the synthesis of both insulin chains was caused by the successful regeneration of 1–2% insulin activity from the separated and inactive A and B chains by Dixon and Wardlaw [41]. The last sentence in their publication was like reading an appeal to start the chemical synthesis of insulin.

> Even with the present yield, it can be said that if chemically synthesized A and B chains were available in mg amounts it should be possible to obtain insulin by the above method and thereby provide the terminal step in the total synthesis of a protein with biological activity.

Several groups of peptide chemists began to focus on synthesizing insulin. My team comprising Meienhofer, Schnabel, Brandenburg, Klostermeyer, Okuda, Zabel, Sroka and Bremer [6] was the first to achieve the combination of synthetic A and B chains of insulin with biologically active material.

Another team was led by Katsoyannis [42] at the Biochemical Institute of Pittsburgh and a third group of Chinese scientists was led by Wang [43] at the Institute for Biochemistry of the Academia Sinica in Shanghai. Jakubke and Jeschkeit [44] have summarized our strategy of synthesis and noticed that the syntheses of the American and Chinese teams differ in methodical details only (Figure 10).

Insulin research in Aachen was no longer a spin-off of wool and fibroin research as in Heidelberg. It became an independent research project of the new insulin division of the Wool Research Institute with Meienhofer as its first head. First synthetic insulin chains served as model proteins in various wool research projects. With my Ph.D. students I was working on the improvement of chain combination methods, as well as on a new tactics of *S*-protection by coupling symmetric cystine peptides (Figure 11).

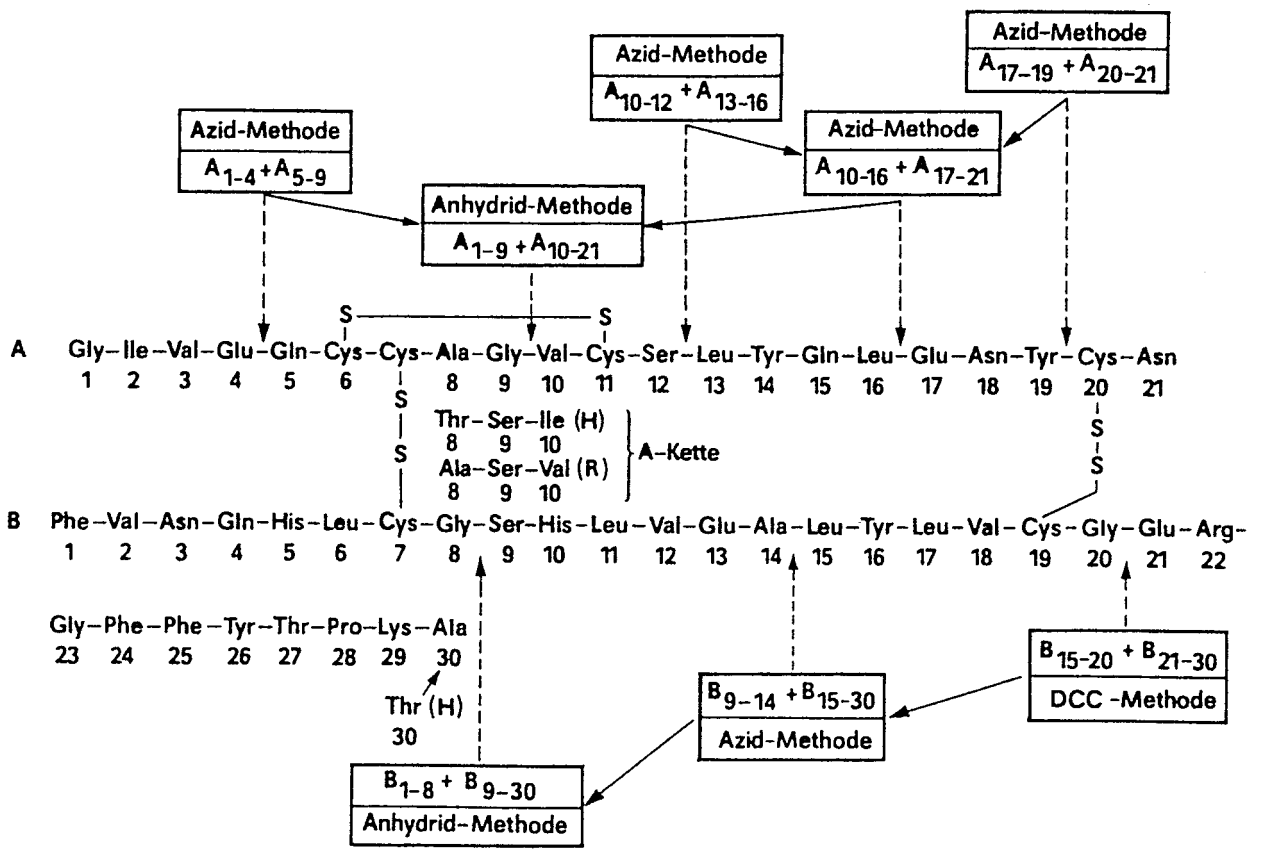

Figure 10 Strategy of Zahn, Meienhofer and Schnabel (from Jakubke and Jeschkeit [44]) for the synthesis of sheep insulin.

Although I have to restrict this review to my early journey to insulin in the 1950s, I feel it appropriate to refer to the review article by Weitzel [47], who has documented the publications from the Institute on insulin and proinsulin until 1977.

THINKING BACK TO MY EARLY RESEARCH WORK

In this last Section I will make some personal remarks on my present thoughts on my work on chemical cross-linking, oligomers, fibroin peptides and insulin synthesis. Which one withstood the test of time and which is of historical interest only?

The *chemical cross-linking* of proteins remains one of the site-directed approaches in protein chemistry [48,49].

Certainly our first cross-linking molecules FFDNB and FF-sulfone are only rarely used, but active esters of dicarboxylic acids are still often applied to the synthesis of insulin dimers [50].

As far as our work on *oligomers* is concerned, I am pleased to see that our results on the onset of chain-folding at the nonamer of ε-aminocaproic acid (Figure 8) have been amply corroborated by Atkins and his associates [51]. They showed that the once-folded structure for the 9-amide chains represents the onset of folding in their monodisperse nylon 6 oligoamides synthesized by Brooke *et al.* [52].

While we synthesized *fibroin peptides* to find the onset of the β-structure, we could not foresee that this research field would still be a focus of interest in material sciences [53] as well as in molecular medicine. The self-assembly of β-sheet domains results in the formation of ordered fibrillar structure as a common feature of various medical disorders [54].

My present thoughts on the *insulin synthesis* are mixed: on the one hand it is of historical interest only. Correspondingly, the Aachen insulin synthesis has been exhibited since 1995 in the 'Deutsches

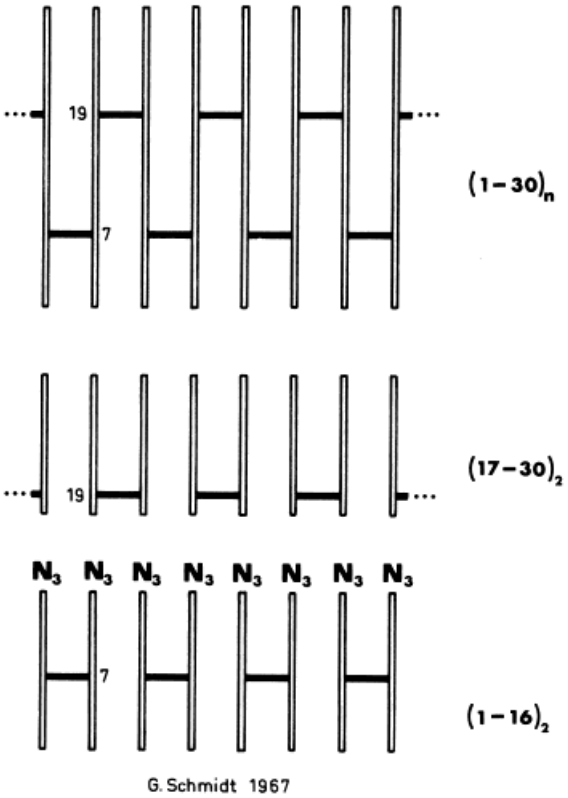

Figure 11 The synthesis of the insulin B chain polymer cross-linked via disulfide bonds [45,46].

Museum Bonn' [55]. The exhibit consists of written explanations, plots of the structure of insulin, small samples of some of the larger A and B chain peptides and the laboratory diary of Bremer with handwritten entries on all steps of his synthesis of the complete A chain, the combination with the synthetic B chain of Schnabel and Meienhofer and the data on the biological activity [56]. Strictly speaking, the first insulin syntheses were already outdated in 1974 when Rittel and his associates published their total synthesis of human insulin involving directed formation of the three disulfide bonds at different stages of the fragment-condensation approach [57].

On the other hand, I have strong arguments against negative statements on the practical utilization of the chemical syntheses of insulin. The work in the 1960s did not only open the way to the synthesis of many other proteins even through improved alternative methods, but made insulin a widely explored molecule for chemical and enzymatic semi-syntheses. Here it is to be remembered that the transpeptidation of porcine into human insulin has made human insulin available for the treatment of diabetes since 1982. Jonczyk and Gattner's publication [58] on this subject is evidence for the industrial application of fundamental insulin research.

Acknowledgements

I would like to express my warmest thanks to Professor Hartwig Höcker, Director of the German Wool Research Institute at the University of Technology in Aachen, for his continuous encouragement and support.

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