MEETING REPORT

JOINT BIOCHEMICAL MEETING

of the Société Belge de Biochimie - Belgische Vereniging voor Biochemie and the Gesellschaft für Biologische Chemie

on

PEPTIDES AND PROTEINS

and

ANTIGENS AND IMMUNOGENICITY

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1. Introduction

The meeting took place in Liège on the 14–16th January, 1971, sponsored by the Université de Liège, Behringwerke AG, Division Pharmaceutique de l'U.C.B. and Janssen Pharmaceutica n.v. Its excellent organisation was due to the efforts of Claude Liébecq. On behalf of the organising societies, the neighbouring universities of Aachen and Liège undertook to prepare the programme, which thus reflected primarily the interests of these groups. The great number of abstracts submitted necessitated the running of simultaneous sessions. For this reason, only the papers read in the *Peptides and Proteins* Section will be reported here and the communications given on *Antigens and Immunogenicity* will be dealt separately [1].

As Marcel Florkin emphasised in his welcome address, by coming to Liège the German biochemists were following the steps of Theodor Schwann, who not only discovered pepsin in Berlin but was also the co-founder of the cell theory in association with Schleiden [2]. It was thus symbolical that this joint meeting should take place in the building where his memorial stands.

In view of the widespread field of protein chemistry covered by the programme, the main topics were introduced by invited lecturers to orientate the ensuing discussion: each paper was published in abstract form [3].

2. Peptide synthesis

Theodor Wieland (Heidelberg) reviewed the history of the development of peptide synthesis [4]. He dwelt at length upon the apparent difficulties of synthesising peptide chains either beyond the length of ACTH (39 amino acids) or the size of the insulin molecule (two chains comprising 21 and 30 residues, respectively). Attempts to build up larger proteins led, up to now, to the formation of biologically active compounds at best instead of chemically well defined substances. Using both his own data and those of Bayer, he convincingly demonstrated the rapid and inevitably growing increase in the costs of synthesising larger proteins, both in terms of time and money. The reason for this is that the multi-step synthetic procedures consist of a series of incomplete reactions. The

automation of individual steps could only simplify the synthetic process without improving its end results, neglecting an important element of peptide chemistry, i.e. the uniqueness of each amide bond. In the author's own laboratory, automated synthesis brought satisfactory results up to decapeptides only, which are still accessible to further purification. To synthesise larger molecules, both the improvement of present methods and the development of new, preferably enzymatic, techniques are required.

The present division of peptide chemists into the followers of Merrifield and those who still use conventional methods will probably disappear sooner or later. Several peptides usually synthesised by conventional techniques could certainly be assembled more simply and automatically on polymer carriers. Probably the partisans of the peptide-synthesiser would also be more critical towards their products. Thus, S. Hörnle (Tübingen), using ^{14}C-labelled isothiocyanate to indicate unchanged amino groups, showed that with the Merrifield method at best only a 99% conversion is possible, even if there is no steric hindrance to the coupling. Actually, the amino acid derivatives used were converted to the extent of 93.4–98.8%. It seems essential to study each reaction in advance if optimisation of the automated synthesis is to be achieved. This was done by A. Loffet (Brussels) for the first step of solid phase synthesis, the esterification of the carrier. Accordingly, he was able to improve the results by using tetramethyl ammonium hydroxide instead of triethylamine.

K.P. Polzhofer (Hamburg) applied the original Merrifield method to the synthesis of a pentadecapeptide split specifically by renin. By using gel filtration and ion-exchange chromatography he was able to purify the synthetic product to such an extent that it appeared to be homogeneous in several test systems. W. Voelter (Tübingen) reported on a joint project of four German and American laboratories involving the solid phase synthesis of immunoreactive pentadeca and eicosipeptides with TMV sequence. Unfortunately, instead of the desired compounds a series of compounds were obtained. However, the main components were neither immunologically nor chemically identical with the native peptides. Therefore, the author recommended the solid phase method only for synthesis of peptides comprising 10 or less amino acids. It is hoped that one of the co-authors, J.D. Young (Berkeley) would include this result in the next edition of her well-known book [5] on solid phase peptide synthesis.

U. Weber (Tübingen) discussed an improved synthesis of the insulin A-chain with this method. The removal of *S*-protecting groups by sodium in liquid ammonia was an inadequately solved problem, because of the 4 cysteine residues of the A-chain. This was bypassed by using 6 *S*-alkylmercapto groups, which could be removed by sulfitolysis at the end of the synthesis. Subsequently, the chain was purified as an *S*-sulfonate and recombined with native insulin B-chain according to Katsoyannis. In control experiments on the recombination of native insulin chains, a yield of 40–60% biological activity based on B-chain was achieved. When A-chains synthesised with the aid of *S*-benzyl groups and sodium reduction were used for recombination, the yield of this preparation was only 10–15%. Using *S*-alkylmercapto protecting groups, however, the yield could be improved to 20–30%. The only explanation for the fact that, despite extensive purification of the synthetic chains, only a product of decreased biological activity could be obtained is the presence of chemically closely related by-products in the preparation. This seems to be characteristic of the Merrifield synthesis.

Hence W. König (Hoechst, Frankfurt) carried out the synthesis of human insulin A-chain by conventional methods allowing the purification of intermediate products. In this work, trityl groups were again used for the critical S-protection; their removal by iodolysis seems to be better than the usual acidolytic cleavage previously applied. Of particular interest in his approach was the complete blocking of all glutamine side amino-groups, making the chain highly soluble in organic media. Thus, purification became much easier. Contrary to current practice, however, some side chains were not protected throughout the synthesis but deblocked in the course of the process. A novel feature of this method was the splitting of *o*-nitrophenyl residue from the amino groups in neutral medium through pyridiniumbromide/indol. This enabled a ready combination of side chains with acid-labile protecting groups. Thereupon H. Klostermeyer (Aachen) pointed out that this method had also been developed in the Deutsches Wollforschungsinstitut and was also suitable for the cleavage of *N*-trityl and bisphenylisopropyloxy-carbonyl groups.

V.K. Naithani and P. Fehrenbach (Aachen) reported the synthesis of fragments for the build-up of the proinsulin molecule. Even the synthesis of medium-sided fragments necessitated repeated modifications of the overall plan and the total synthesis is still beset by considerable difficulties. However, the question of S-protection was solved by synthesising fragments in which the B7–B19, A6–A7 and A11–A20 cysteine residues are cyclicised to form cystine. At present, the methods for joining these rings together are being investigated.

H. Klostermeyer (Aachen) presented his studies on the synthesis of fragments forming the viral coat protein of phage fd. The main difficulties of this project are, in the first place, the lack of biological activity. Hence, the synthesised protein could not be tested by bioassays but only by chemical techniques. Second, a poor solubility of both the complete protein and some of its parts, unparalleled so far in peptide chemistry, requires new preparative procedures.

The synthesis of new peptides and proteins through the use of native fragments is a rapidly developing new branch of peptide chemistry. Since insulin is thoroughly investigated and readily amenable to analysis, it appears to be particularly suitable for this kind of work. However, as shown by R. Geiger (Hoechst, Frankfurt), surprising results may be obtained. Contrary to the literature, the reaction of insulin with excess tertiary butyloxycarbonylazide did not lead to the acylation of all amino groups in aqueous alkaline medium but rather to a specific diacylation of the amino groups of A1 and B29 residues. Thus, it became possible to modify the third amino group with another reagent, e.g. phenylisothiocyanate, giving rise to desPhe^{B1}-insulin following acid treatment. By repeating this process, crystalline desPhe^{B1}-desVal^{B2}-insulin and eventually desPhe^{B1}-desVal^{B2}-desAsn^{B3}-insulin was obtained. If pyridine is used as a solvent, the specificity of acylating reactions disappears.

Similar observations were made by D. Brandenburg (Aachen), studying the effect of activated esters on insulin. In addition to the expected aminoacylations, cross-linking also developed to an increasing extent, dependent on the nature of the solvent. As demonstrated with ^{14}C-acetic acid-*p*-nitrophenylester, first a mixed anhydride was formed between the insulin carboxyl groups and the acyl components of the activated ester. Subsequently, this was transformed into inter- and intrachain cross-linkages by aminolysis.

T.R. Csorba (Aachen) showed that cross-linking also occurs during iodination of insulin. The cross-linked material was separated from the monomer by gel chromatography in 33% acetic acid. However, in alkaline solution, even at pH 7.4, the monomer was partially converted into a cross-linked derivative. This phenomenon might shed some light on the different behaviour of iodoinsulin in vivo, as compared with the native hormone.

H.-G. Gattner (Aachen) has extensively investigated the nitration of tyrosine residues in the insulin molecule with the aid of tetranitromethane. Whereas it was already known that nitration also leads to cross-linking of insulin, the characteristics of monomer nitro-insulins at different levels of substitution and the reaction sequence of the individual tyrosines were studied first by the author. These products could be fairly well separated by ion-exchange chromatography and characterised subsequently by enzymatic degradation. The nitration of each tyrosine residue proceeds at a different velocity and also causes a marked reduction in the immunological reactivity.

3. Protein structure

This topic was introduced by a lecture of P. Jollès (Paris) on the Principles and Methods of Structure Analysis [6]. Although the primary structure could be elucidated in proteins with hundreds of amino acid residues, sequence analysis has not yet become a routin procedure. Automation through the sequencer is a useful tool but it has serious limitations. The rapid automatic identification of degradation products remains to be solved. At present the best method seems to be gas chromatography. On the other hand, the combination of mass spectography with computer analysis is just at the beginning of its development for these purposes. In the future, primary structure might be determined simultaneously with structures of higher order using X-ray diffraction studies but this is not yet feasible. It is urgently necessary to find a method allowing degradation of proteins at the *C*-terminal and to develop the enzymatic techniques further. In this respect, remarkable progress has been achieved through the covalent binding of enzymes to synthetic carriers. The improvement of enzymatic methods would be of particular significance in the elucidation of glycoprotei

structure. It should not be forgotten, however, that often the brunt of the work in characterising a protein is not the sequence analysis but rather the isolation and purification procedures.

This was followed by the lecture by M. Florkin (Liège) on the paleoproteins found in fossils. It was demonstrated that fossils over several million years old also contain proteins. It would certainly be important to analyse this material beyond the simple determination of amino acid content. Florkin proposed that analytical criteria for proteins could be applied to the investigation of geological processes, even if the extracted material is not sufficient for the proper characterisation of this protein. This was in line with the paper of W. Thiemann (Jülich), who studied the racemisation of a dipeptide kinetically in order to establish guidelines for the interpretation of biological and geological experiments.

A methodological contribution was given by S. Bricteux-Grégoire (Liège). Proteins were electrofocused in polyacrylamide gels, the ampholyte was removed by extraction with trichloroacetic acid, followed by the dansylation of protein in the gel. After total hydrolysis, the dansylated amino acids could be determined by a special technique. The ensuing discussion was centered on the question: to what extent does the separation pattern of a protein in acrylamide electrophoresis and focusing reflect the actual inhomogeneity, and to what extent are artefacts introduced by these methods? If this cannot be resolved unequivocally, the occurrence of artefacts should be assumed.

W. Voelter (Tübingen) discussed the possibilities of applying ^{13}C-NMR spectroscopy to peptide and protein chemistry. The use of the pulse-Fourier technique might allow a study of higher molecules, particularly on the cysteine–cystine redox equilibrium in proteins.

The paper of G.F. Domagk (Leuven) dealt with the crystalline beef muscle fructosediphosphate aldolase, introducing communications on individual proteins. Whereas the molecular weight and kinetic data agree with that found in the rabbit muscle enzyme, the amino acid composition and immunological behaviour as well as the α-helix content show significant differences.

C.F.A. Bryce and R.R. Crichton (Glasgow) reported, in two communications, on the molecular weight and cyanogen bromide peptides of horse spleen apoferritin. Contrary to previous assumptions, the molecular weight of the subunits lies around only 18,500 daltons instead of 25,000–27,000 daltons. The protein is composed of 24 instead of 20 subunits and bromcyan treatment yields 4 fragments.

H.G. Wittman's group (Berlin) studied an extremely complex system from *Escherichia coli*, presenting three papers given by W.Rombauts, B. Wittman-Liebold and G. Stöffler on the characterisation of a ribosomal protein. 55 proteins were isolated from the 70 S unit in pure form, in amounts of 0.5–1 μmoles. Amino acid composition and partial sequence determinations suggested the presence of different components. Some of these could be assigned to a given function, thus allowing chemical and immunological comparison with proteins from known strains and mutants of *E. coli*. This opens the way to another field by using a new separation technique [8] for the investigation of individual ribosome components.

K. Han (Lille) carried out comparative studies of sheep heart myoglobin with the protein from beef heart studied previously by him. In the partial sequenc presented, 114 of the 118 residues were found to be identical. G. Buse (Aachen) discussed an unusual haemoglobin from insects, having a chain length of only 136 amino acids. In this sequence, some of the parts, which according to Perutz et al. [9] are responsible for the alkaline Bohr effect of haemoglobin, are missing. Since the insect haemoglobin also shows this effect, its mechanism ought to be reconsidered.

Finally, G. Hennen (Liège) reported on the primary structure of a cyanogenbromide fragment with 21 amino acid residues from the *C*-terminal of the α-subunit of porcine and bovine luteinising hormone.

4. Collagen and structural proteins

The introductory lecture was given by K.A. Piez (Bethesda) on the structure of collagen and elastin [10 The collagens investigated comprise three peptide chains, each containing about 1000 amino acid residue Two chains, termed α1, are identical, varying very littl in composition from different sources. The α2 chain, on the other hand, shows wide species variations in the *N*-terminal region. The 10–20 amino acids of this region, for the three chains of the molecule, show nonhelical regions of lysine and hydroxyllysine residues which are partially transformed enzymatically in

aldehyde (allysine and hydroxyallysine). These residues react with each other through ϵ-amino groups to form cross-linkages between the individual chains of the molecule.

Although the collagen of skin, bone and tendon is coded by the same gene, the composition of the cartilage component is different in having 3 identical peptide chains of type $\alpha 1$. Elastin has a far less ordered structure than collagen, forming desmosine and isodesmosine on cross-linking of the chains. These are not present in collagen.

Further information on the collagen molecule came from K. Kühn's laboratory in Munich. M. Stark reported on the size and structure of fragments obtained by means of bromcyanide from calf skin collagen and also from the *C*-terminal of its $\alpha 1$ chain. This region is also not helical. K. v.d. Mark, in an elegant analysis, was able to correlate the amino acid sequence with the cross striation pattern of collagen.

A very good example of collaboration was shown in the papers of 4 Belgian groups. First, R. Hanset (Brussels) discussed the veterinary aspects of a newly described skin disease in calves, called dermatosporaxis. The animals suffer from a dramatic fragility of the skin, caused by a defect of connective tissue. Through the follow-up of 84,866 births, the disease was found to be genetically determined.

L.J. Simar (Liège) dealt with the ultrastructure of the skin as an anatomist demonstrating anomalies in collagen fibers without involvement of the small vessels and fibroblasts. A. Lenaers (Liège) reported on the fractionation and chemical characterisation of the collagen isolated from the skin of sick calves. In addition to the normal components, this collagen also contains new ones, characterised through the occurrence of cysteine and cystine. K.A. Piez in his comment pointed out that collagen would probably be first synthesised in a transport form and possibly in this case the conversion from transport form into proper collagen was disturbed.

Actually, the defect of the connective tissue does not appear to be due to a simple alteration in the sequence of the synthesised collagen. R.J. Winand (Liège) fractionating glycosaminoglycane and proteoglycane from both healthy and pathological skin found distinct changes, both qualitatively and quantitatively. Since then dermatosporaxis was also observed in other places and this might allow the clarification of the links between the biosynthesis, structure and function of collagen.

R.J. Winand has also presented another communication in the rather difficult field of glycoproteins. He succeeded in fractionating the structural glycoproteins of the renal basement membrane and found two types of oligosaccharides in each fraction lying besides each other on the peptide chain.

U. Groeschel-Stewart (Würzburg) has also found a glycoprotein in actinomyosin and myosin from human myometrium. This had the characteristics of a ground substance and could be localized in the tissues as well as in human amyloma protein with fluorescein by using specific antibodies. Uterine myosin has an amino acid composition different from that in myosin from skeletal muscle. However, antibodies raised against the latter show only a low degree of specificity.

J. Lowy (Aarhus) discussed X-ray diffraction and electron-optical studies on smooth muscle aimed at getting information on the structure of myosin in the relaxed and active state. In fact, the intensity of myosin reflexion is rather limited and this is further reduced on contraction, indicating a ribbon-like structure with elements of different periodicity. The clarification of the molecular mechanism of action for the actinomyosin/myosin system certainly requires further investigation.

5. Immunoglobulins

One of the highlights of the symposium was the lecture of Norbert Hilschmann (Göttingen) on immunoglobulins which was given in a depth and detail which is rarely heard at meetings. First he gave a clear classification of the immunoglobulins. In this, Hilschmann used the concepts of isotype, allotype and idiotype in order to explain the manifold roles of these compounds on a relatively simple molecular basis. In particular, this made it possible to present convincing arguments in favour of the earlier assumption [11] that each immunoglobulin is determined genetically through the fusion of two genes, of which one is constant and the other gene is variable. This lecture provided the link between the two main sections of the meeting.

J. Rudinger (Zurich) in his closing remarks discussed the current trends in protein chemistry. Althoug

there is an increasing tendency to use automated equipment, success does not necessarily follow. The proteins still present many puzzles and however useful the machines might be to clarify these, they do not always suffice to this end. Nevertheless, there is a rapid progress as shown by the papers presented on insulin chemistry, reflecting a much more critical approach than before. Another encouraging aspect is the development of multi-team cooperation that allows the study of individual subjects from many angles. In Rudinger's opinion, which is certainly shared by the participants, this meeting was both useful and rewarding.

References

[1] O. Westphal and E. Rüde, FEBS Letters, in preparation.
[2] M. Florkin, Naissance et Déviation de la Théorie Cellulaire dans l'Oeuvre de Théodore Schwann (Hermann, Paris, 1960).
[3] Z. Physiol. Chem. 352 (1971) 1.
[4] Th. Wieland, C. Birr and F. Flor, Liebigs Ann. Chem. 727 (1969) 130.
[5] J.M. Stewart and J.D. Young, Solid Phase Peptide Synthesis, (Freeman, San Francisco, 1969).
[6] P. Jollès, Angew. Chem. 81 (1969) 244.
[7] M. Florkin, A Molecular Approach to Phylogeny (Elsevier, Amsterdam, 1966).
[8] E. Kaltschmidt and H.G. Wittmann, Anal. Biochem. 36 (1970) 401.
[9] M.E. Perutz, H. Muirhead, L. Mazzarella, R.A. Crowther, J. Greer and J.V. Kilmartin, Nature 222 (1969) 1240.
[10] K.A. Piez, Ann. Rev. Biochem. 37 (1968) 547.
[11] N. Hilschmann, Naturwissenschaften 56 (1969) 195.