REFLECTIONS ON CONTROL OF PEPTIDE SYNTHESIS*)

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Three principal possibilities for the analytical control of the effectiveness of coupling and deprotection steps during the solid phase peptide synthesis are the following: 1. analysis on samples 2. determination on the whole batch of resin-bound peptide. 3. measurement on the liquid phase. These different methods are discussed in detail.

In the past few decades a dramatic and ever increasing broadening of our insight into living matter has taken place. The growing information about hormones, enzymes, and other susbstances of a protein nature in the normal and the pathologic organism has for various reasons augmented the demand for synthetic peptides for biochemical and biological studies or for use in the clinic.

To the young generation, peptide synthesis certainly appears to be a rather modern invention. The fact is, however, that it dates back to 1882 in Leipzig, when the 25-year-old THEODOR CURTIUS, assistant to professor HERMAN KOLBE, reported the first synthesis of a peptide [1]. The result was achieved inadvertently by benzoylation of silver glycinate in an attempt to clarify the structure of hippuric acid. He thus carried out the first mixed anhydride coupling resulting in the formation of benzoyl-glycylglycine. About 70 years later CURTIUS' experiment inspired THEODOR WIELAND to initiate his studies of mixed anhydrides. As is well known, EMIL FISCHER also contributed to the origin of peptide synthesis, and in 1907 he was able to report the synthesis of a leucine and glycine-containing peptide consisting of 18 amino acid residues [2].

Elemental analysis was the only method of control at hand for CURTIUS and FISCHER, and is today — almost 100 years after the first reported peptide synthesis — still a fundamental method for characterization of low molecular weight peptides.

The real challenge to peptide synthesis was launched in 1955, when SANGER reported the complete amino acid sequence of bovine insulin [3]. Peptide synthesis can in no way be said to have caught up with the rate with which larger and larger proteins are being sequenced.

Two main principles are applied in the attempts to synthesize larger peptides by organic chemical means, which involves the use of organic solvents. In one case,

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called synthesis in solution, larger chains are obtained by condensation of fragments. In another case, the synthesis is performed on a polymeric support, and the growing peptide is covalently bound to the polymer, which is generally insoluble. This method is termed solid-phase peptide synthesis. In this case, stepwise elongation is usually preferred.

In both synthesis in solution and in solid-phase synthesis, the chain elongation is carried out from the C-terminal amino acid in order to avoid racemization. The advantage of synthesis in solution — whether by stepwise chain elongation or by fragment condensation — is that in principle an intermediate purification is possible, especially if the intermediate products can crystallize. An advantage of fragment condensation compared to the stepwise approach is that the isolation is in principle facilitated by the pronounced difference in molecular size between the reactants and the product.

The yield of a single reaction in synthesis in solution is thus not crucial for obtaining the final product as long as isolation of the desired product is possible, whether this be an intermediate or the final product. Serious obstacles, however, fairly soon set a limit as to how large molecules can be built by synthesis in solution, restricting the considerations to the synthesis of molecules with a predetermined sequence and not considering polyamino acids.

One of the limitations to the use of synthesis in solution is the fact that the solubility of the protected peptides may be low and is decreased by increasing chain length. The coupling reaction in such a case is therefore carried out with the reactants partly dissolved, and the phrase 'synthesis in solution' is then not a very correct one.

The low solubility also restricts the use of an excess of the acylating fragment, and the coupling between large fragments is hampered significantly by the size of the molecule and proceeds slowly. If glycine is not the C-terminal amino acid in the activated fragment, there always exists a danger of racemization, due to the adjacent peptide bond. Finally an economic consideration is involved, as peptides are always expensive.

The fundamental advantage of fragment condensation is that the amino acid sequence in the final product is the desired one, as it corresponds to the fragments assembled.

The evidence demonstrates that it is possible to obtain reasonable quantities of products of high purity, containing 30—40 amino acid residues, but it should be stressed that the results have always required a very great effort. The synthesis of sheep insulin by ZAHN and coworkers [4] and later of ox insulin by a Chinese group [5], in amounts which were sufficient for crystallization, are certainly the most impressive achievements in classical peptide synthesis.

In the solid-phase technique, the isolation of the reaction product is carried out by simple filtration, and the procedure of chain elongation can be automated. Whether all solubility problems are circumvented, however, is open to discussion. Thus, it cannot be excluded that the use of certain solvents might result in a conformation of the polymer-bound peptide unfavorable to the process of chain elongation. The shortcomings of the solid-phase technique are inherent in the very principle. Thus, shorter peptides lacking one or more residues will be accumulated if the addition of an amino acid is not performed with a yield of 100%. Furthermore, damage which takes place to a slight degree in each step will be aggravated due to the repe-

titive procedure. Difficulties may also be encountered in the cleavage of the final product from the polymer. The application of the technique therefore easily results in a very complex mixture of peptides, from which it is often impossible to isolate the desired product.

In the synthesis of large peptides either in solution or by the solid-phase technique, mainly naturally-occurring substances have been prepared. In these cases a comparison of the synthesized product with the authentic substance is possible. When dealing with complex reaction mixtures one is lost, unless additional information can be obtained. In fact, with large molecules we must be content with an examination of biological or biochemical activity. However, such an activity may be due to the presence of shorter sequences or other byproducts. In the case of the synthesis of large molecules possessing only slight or no hormonal or enzymatic activity at all, no adequate proof of the achievement of the synthesis is at present obtainable.

The analytical problems to cope with in peptide chemistry when not considering rather small peptides are complex, and the complexity increases with increasing molecular size. The above-mentioned considerations only dealt with the process of chain elongation. For both the mentioned synthetic principles, however, common problems exist regarding protection and deprotection of functional side-chain groups, due to the fact that no procedure is at hand for a specific formation of the peptide bond between the α-amino and α-carboxyl groups.

Amino acid analysis is widely used in the control of the synthesis and is often considered a trivial matter of routine. In fact, however, even when we are dealing with small proteins, the shortcomings of the technique are evident. Several hydrolyses have to be performed with and without additives. In order to achieve usable results for some amino acids, it may even be necessary to correct the values through comparison with the values for these amino acids obtained by analysis of a closely-related protein with known sequence.

The introduction of the solid-phase technique by MERRIFIELD [6], LETSINGER and KORNET [7] was received with enthusiasm. The reasons were the facility of isolation of the resin-bound product, the relative ease of automation and the possibility of scaling up the synthetic procedure. The results, however, have not fulfilled the expectations. The ease with which the procedure is carried out has led many astray, tyring to synthesize much larger molecules than the technique allows. For the synthesis of chains of up to approximately 30 amino acids, the technique has turned out to be valuable in several cases, especially where a rigorous purification is possible, as in the case of cyclic peptides. By fragment condensation on the resin, using Pro-Pro-Gly SAKAKIBARA *et al.* prepared $(Pro\text{-}Pro\text{-}Gly)_{10}$. A sufficient purity for crystallization of the final product was obtained [8].

Calculations have been carried out by Bayer et al. of the required minimum yield of the single increment for a certain yield of the desired peptide. The calculations are based on the C-terminal polymer-bound amino acid [9]. Provided there is a yield of 99% for the addition of each amino acid, the total yields of the desired polymer-bound peptide chains for human growth hormone, consisting of 190 residues, and for the A-chain of insulin, consisting of 21 residues, are 15 and 82%, respectively. However, low yields in some steps of the synthesis, and damage occuring during the process of chain elongation or through the cleavage of the product from the resin or the deprotection, often result in a low total yield and lead to a mixture of closely-

related substances, from which it may be impossible to isolate the desired peptide. Cleavage of peptide from the resin during the synthesis leads to a reduction of the total yield, but it does not complicate the isolation procedure.

It is evident, therefore that unless at least the process of chain elongation is brought under control, the solid-phase technique is not to be expected to be generally applicable. The ideal solution for the monitoring of the process would be an automated non-destructive continuous procedure for determination of the number of free amino groups liberated by cleavage of the α-amino protection group, and the number of peptide bonds formed during each coupling. This would allow us to follow the time-course of the reaction and determine the final yields. No such method exists, however.

An excellent survey of the literature dealing with analytical procedures applied is solid-phase syntheses up to 1973 is given by HIRT *et al.* in Chemistry of Polypeptides, Essays in Honor of Leonidas Zervas [10].

The developed methods most suited for monitoring are based on determination of the number of free amino groups after the coupling and after the cleavage of the protection group. Indirect measurement has been carried out by UV monitoring of the liquid phase after deprotection or coupling.

Three principal possibilities exist for carrying out an analytical control: 1) analysis on samples; 2) determination on the whole batch of resin-bound peptide; 3) measurement on the liquid phase (Fig. 1).

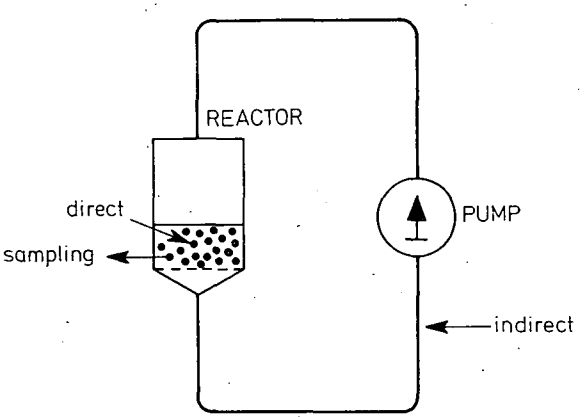

Fig. 1. Different principles for analytical control of solid-phase synthesis [10*a*]

1. Sampling

Several analytical procedures have been applied for analysis on samples, thus for example automatic Edman degradation, mass-spectrometry, amino acid analysis and Schiff base formation. For detection of residual amino groups after the coupling, ninhydrin was used by KAISER *et al.* [11]. As little as 5 μmole/g resin can be detected. It is difficult, however, to evaluate whether the test is positive or negative, especially if the resin is colored during the synthesis. The use of fluorescamine was introduced

by FELIX and JIMENEZ [12]. By this method as little as 0.6 μmole/g resin is claimed to be detected. The experience up to now is rather limited and interference from non-specific binding seems to reduce the value of the test. With ninhydrin N-terminal proline and β-benzyl aspartate only give rise to an unsatisfactory color development; with fluorescamine no reaction takes place with proline.

Quantitative analysis based on samples is complicated, because it must be known with great accuracy how much the sample amounts to of the total quantity of peptide resin. In synthesis on a minor scale this can be determined by drying the total mount of resin and carrying out the analysis on an aliquot. In principle this determination could be facilitated by the use of an internal standard, also making it possible to monitor a synthesis on a large scale, but up to now this appears not to have been achieved.

Quantitative measurements of free amino groups by titration with perchloric acid on samples have been carried out by SCHOU [13] (Fig. 4). The starting amount of resin was 25 g, and 1 g was withdrawn after each deblocking and coupling cycle. Regarding the perchloric acid titration, a closer description will be given in the following.

The development in high performance liquid chromatography, HPLC, has resulted in a highly efficient technique for analyzing peptide preparations. Coupling with a fluorescent compound may facilitate the detection of peptides with a free amino group [13*a*]. Characterization by HPLC is to be considered as a standard procedure in peptide synthesis for investigation of the homogeneity of the end product, even regarding chirality [13*b*].

The possibility of carrying out the separation on a small scale makes the method applicable for monitoring of peptide synthesis, however, by solid phase synthesis, taking into consideration artefacts deriving from the cleavage of the peptide from the resin. The fact that small scale preparative fractionation is easily performed allows a closer examination of the various fractions, for example by mass spectrometry [35].

2. *Determination on the whole batch of resin-bound peptide*

MERRIFIELD [14] introduced a procedure for the estimation of the amount of free amino groups, after cleavage of the Boc groups with N HCl/HOAc. The resin was treated with triethylamine in dimethyl formamide, the filtrates were collected and the chloride was determined by the Volhard procedure. BAYER *et al.* [15] used this procedure to monitor the synthesis of apoferredoxin, 55 residues, and demonstrated a significant decrease in free amino groups during the synthesis. DORMAN modified the procedure so that it could also be used for measurement of residual free amino groups after the coupling [16]. This was achieved by using pyridine hydrochloride for conversion of free amino groups into the hydrochloride. The hydrochloride is removed by triethylamine and the chloride is determined in the collected filtrates.

GISIN [17] used picric acid for protonation of the free amino groups. After displacement of the bound picric acid by diisopropyl-ethylamine, the amount was determined photometrically. This procedure has been automated [18]. However, adsorption of picric acid on the polymer seems to create complications.

In our institute we have investigated a procedure for the estimation of free amino groups based on a potentiometric end-point titration with perchloric acid in acetic acid, with the resin suspended in a mixture of methylene chloride and acetic acid [19]. By the applied procedure, the same end-point can be used irrespective of the N-terminal amino acid. This is because the acetic acid protonates the amino groups and is replaced by the perchlorate. It is thus in fact the acetate ions which are titrated and not the amino groups, a so-called levelling effect. As this takes place mainly inside the resin, the titration may proceed rather slowly, usually lasting from 5 min up to 1 hour. The accuracy of the determination, however, is rather high: up to $\pm 0.3\%$ per single determination.

The procedure can be carried out on the entire batch or as previously mentioned on withdrawn samples. The procedure has been included in systems for automated peptide synthesis, and in this case the titration is carried out on the entire amount of resin [20, 21].

It is to be stressed that the method is not specific for amino groups, as other groups may be sufficiently basic to allow a titration under the conditions applied. Thus, the imidazole group of Boc-benzyl histidine is titrated, leading to an equivalent increase in the titration value [22].

As a strong acid such as perchloric acid is used for the titration, the utmost care must be taken to reduce the danger of overtitration even locally in the liquid phase close to and in the resin. Otherwise, cleavage of acid-labile protection groups such as Boc may take place.

The titration must therefore be carried out as an automatic end-point titration with a slow addition of the titrant and adjustment of the titrator ensuring a proportional slowing down of the addition of titrant within the preset proportional band. The stirring must be highly effective.

As mentioned, one of the potential errors of the solid-phase technique is an accumulation of artefacts during the synthesis, such as blocking of α-amino groups leading to so-called truncated peptide chains. By the perchloric acid titration it has been possible to demonstrate blocking of amino groups by impurities present in methylene chloride [23], during coupling with histidine [22] and by residual acetic acid [24]. The last example is rather informative and will therefore be dealt with further in detail.

The experiment concerned the synthesis of a sequence of the cyclic decapeptide antamanide, and was carried out and controlled automatically by a punched-tape controlled synthesizer. Dicyclohexyl-carbodiimide (DCC) was used as coupling reagent and tertiary-butyloxy-carbonyl (Boc) for protection of the α-amino groups. The result was rather disappointing because it seemed completely inconsistent with what was to be expected [20]. However, we have been able to show that the titration reflected exactly what really occurred, and via the obtained information we have been able to improve the synthetic procedure.

In the first attempt, starting the synthesis with coupling of Boc-proline to a proline resin, the titration indicated a pronounced loss of the ester-bound proline due to formation of proline diketopiperazine. A new attempt was then made by coupling of Boc-alanine to a phenylalanine-resin.

As seen from Fig. 2, the total yield was low, approximately 20%, as calculated by subtraction of the value of the Boc-protected N-terminal phenylalanine from the value obtained after cleavage of the Boc-group and the difference compared with

the corresponding value for the C-terminal phenylalanine. It is seen that the decreases in certain positions are more pronounced than in others. In the titration values of the Boc-protected peptide chains an increase occurred, especially after the incorporation of Boc-valine.

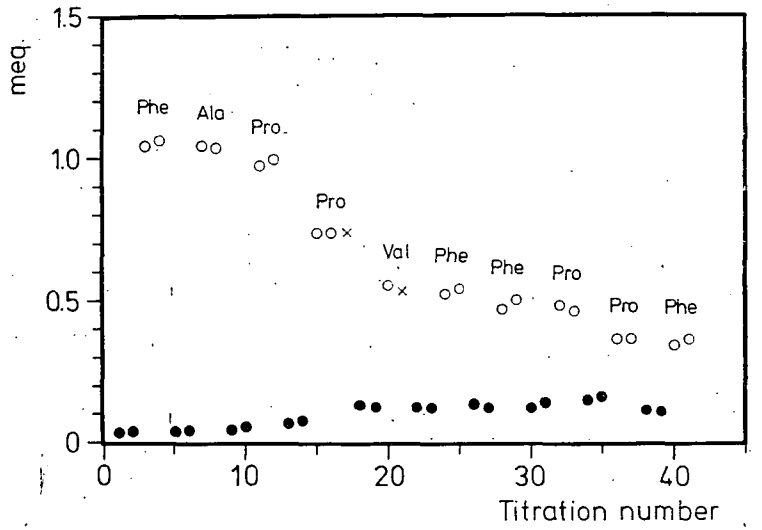

Fig. 2. Titration values determined during the synthesis of the sequence of antamanide: HPhe-Pro-Pro-Phe-Phe-Val-Pro-Pro-Ala-Phe-resin. x indicates that the procedure for cleavage of the Boc group was repeated before the titration. Filled circles before, and open circles after Boc group cleavage [20]

A repetition of the cleavage procedure at the tetra- and pentapeptide stage did not result in higher values, indicating irreversible blocking of the amino groups. If so, a number of shorter peptides should be present in the final product, and amino acid analysis of the total product should differ considerably from the theoretical.

Peptide expected: D_3CCO - Phe - Pro - Pro - Phe - Phe - Val - Pro - Pro - Ala - Phe - OMe

Identified : D_3CCO - Phe - Pro - Pro - Phe - Phe - Val - Pro - Pro -

Shorter peptides identified:
- Ac - Pro - Ala - Phe - OMe
- Ac - Pro - Pro - Ala - Phe - OMe
- Ac - Phe - Phe - Val *
- Ac - Pro - Phe - Phe - Val - Pro - Pro - Ala -

Failure sequences:
- Ac - Pro —— Phe —— Val - Pro *
- Ac - Pro - Phe - Phe - Val —— Pro —— Ala *
- D_3CCO - Phe —— Pro —— Phe - Phe - Val *

*) near detection limit.

Fig. 3. Peptides demonstrated by mass-spectrometry to be present in the product, cleaved from the resin in the experiment shown in Fig. 2 [24].

The presence of the predicted peptides was confirmed by mass-spectrometry, and the blocking shown to be due to acetylation (Fig. 3). The mass-spectrometric analysis was carried out on the entire mixture after deutero acetylation and permethylation, using a technique developed for mass-spectrometric sequence determination of peptide mixtures [25]. By the mass-spectrometry the presence of peptides lacking a proline or a phenylalanine residue due to incomplete coupling is also demonstrated. The reason why only missing proline or phenylalanine residues are demonstrated is probably that these are repeated, and absence of one of them thus leads to the same deleted sequence, and thus to a higher concentration of these sequences.

The reason for the acetylation was that a reactor chiefly made of teflon had been used. Due to the microporosity of teflon, acetic acid was absorbed during the deblocking and titration cycle, and this leaked out during the coupling cycle and was activated by the coupling reagent, DCC. The reason why the acetylation was more pronounced in the coupling of Boc-proline to proline and Boc-valine to proline is simply that these couplings proceed more slowly than the others, allowing more acetic acid to be washed out before the coupling of the derivative was terminated.

Let us return to the Figure illustrating the synthesis. As the blocking occurs during the coupling cycle, theoretically the amount of each amino acid incorporated can be calculated by subtracting the titration value after the coupling from the value after the cleavage of the Boc group. Thus, it is possible to calculate the amino acid composition of the final product — provided loss of peptide does not take place, or to only a slight degree, during the synthesis.

As seen from Table I, A, the amino acid analysis of the resin-bound product shows a fairly good agreement with the titration, thus proving the correctness of the

Table I

Amino acid content of synthetic decapeptide determined by titration and by amino acid analysis [26]

Boc-Phe-0-resin	Amino acid	Theo-retical	Titration	Amino acid analysis			
	Phe	4	4.00	4.00[1]	4.00[2]	4.00[3]	
2.2 g	Ala	1	1.97	1.74	1.88	1.92	
1.00 mequiv (A)	Pro	4	4.23	4.52[4]	4.55[4]	4.73[4]	
	Val	1	0.82	0.76	0.77	0.82	
	Phe	4	4.00	4.00[1]		4.00[3]	4.00[5]
2.9 g	Ala	1	1.20	1.13		1.09	1.01
1.32 mequiv (B)	Pro	4	4.02	3.92[4]		4.16[4]	3.97[4]
	Val	1	0.98	0.97		1.06	1.01
	Phe	4	4.00	4.00[1]		4.00[3]	4.00[5]
6.0 g	Ala	1	1.27	1.08		1.05	1.00
2.74 mequiv (C)	Pro	4	4.04	3.57[4]		3.59[4]	3.96[4]
	Val	1	0.98	0.96		0.92	0.98

[1] Resin-bound product.
[2] Cleaved crude product.
[3] Ether-precipitated product.
[4] Proline (amino acid analysis) corrected for concentration-dependency of calibration factor.
[5] Cyclized peptide (antamanide).

titration values. The experiment clearly demonstrates why amino acid analysis in the synthesis of even small peptides by the solid-phase method may be impossible to interpret. By manually carrying out the synthesis in an all-glass reactor, the total yield of resin-bound peptide calculated from the titration values could be increased to 64% (Table I, B and C). Still, a gradual decrease could be observed in the titration values after deblocking, and also an increase in the values of the Boc-protected peptides after the incorporation of valine. The accuracy was increased, and due to this it was possible to observe on titration of the proline nos. 4 and 9 from the resin that a slight decrease was observed in a second titration, and then constant values in further titrations. The phenomenon must evidently be due to the presence of two adjacent prolines, the N-terminal with a free amino group. It has not been possible to explain this phenomenon, which, however, does not prohibit the coupling reaction.

By reducing the number of treatments by carrying out the titration on withdrawn samples, a further increase to 89% in the total yield of the resin-bound peptide was achieved (Fig. 4) [13]. Here also the abnormality was observed on titration of the two above-mentioned proline residues. By reduction of the number of treatments by the automatically performed synthesis, and using an all-glass reactor, total yields of the same order were obtained by automatic synthesis of other peptides.

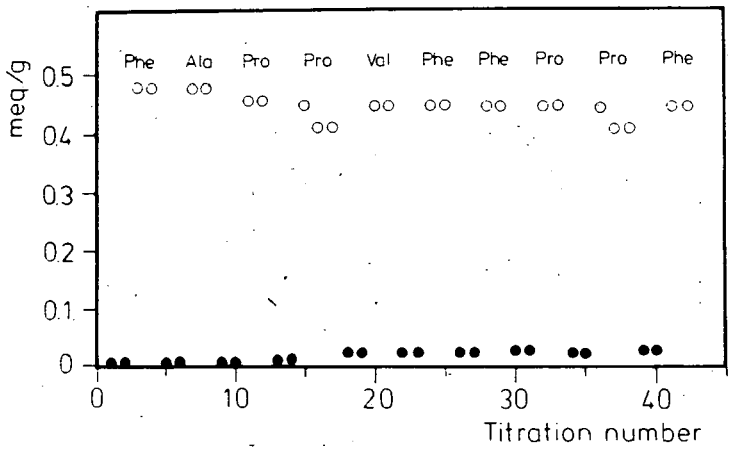

Fig. 4. Titration values on withdrawn samples obtained during the chain elongation in the synthesis of antamanide carried out from the C-terminal Phe (left) to the N-terminal Phe (right). The ordinate values are the numbers of meq calculated per g of Boc-Phe-O-resin. Filled circles before, and open circles after Boc group cleavage [13].

Acetic acid is only removed with difficulty from the resin, and thus could cause terminations by acetylation. Amino acid analysis of the final products, however, indicates that the gradual decrease must be due at least mainly to loss of peptide, resin-bound or cleaved from the resin (Fig. 5).

By replacing proline in position 4 from the resin with leucine, it was ascertained that the increase in the titration values of the Boc-protected peptides was due to proline in this position, as no increase occurred [26]. In a synthesis using ^{3}H-labelled

proline in the same position and ^{14}C-labelled phenylalanine as N-terminal, a binding by alkylation of the proline residue to residual chloromethyl groups on the resin could be demonstrated. As the resulting tertiary amine can be protonated, the apparent increase in the titrations of the Boc-protected peptides was explained and also why the coupling was inhibited to the same extent as the increase in the titration value [27].

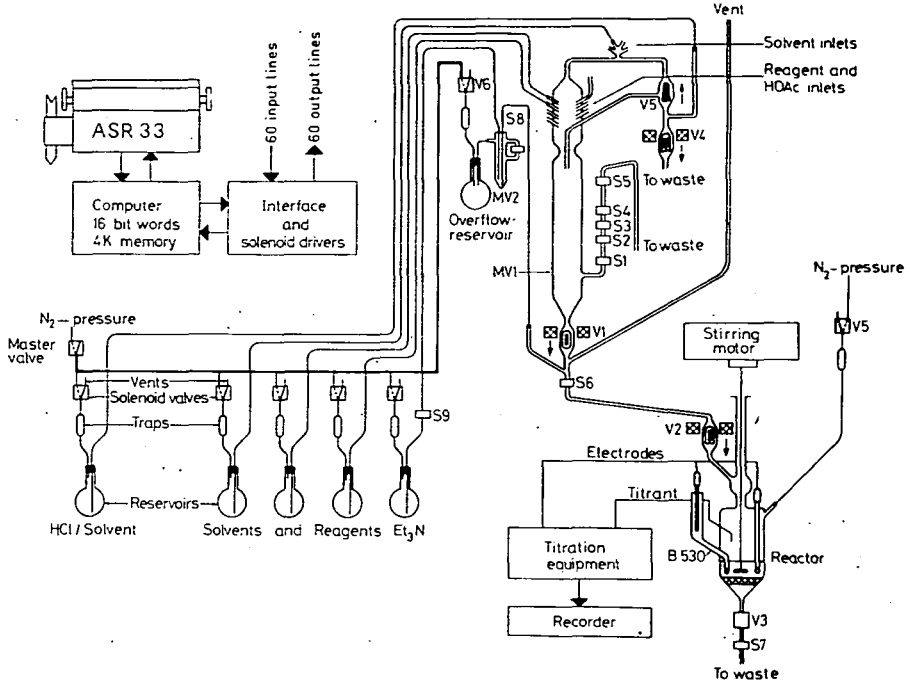

Fig. 5. Schematic drawing of the solid-phase peptide synthesizer. MV1 and MV2, metering vessels; S1 through S9, liquid detectors; V1 through V4, solenoid valves; V5 valve operated by fluid pressure in the line. → indicates flow direction for open valve [30].

The esterification of the first amino acid to the resin was carried out according to LOFFET [28], using the tetramethylammonium salt of Boc-Phe, which leaves some chloromethyl groups intact. If the later-published esterification procedure of GISIN [29], using the cesium salt of Boc-phenylalanine, had been used, no residual chloromethyl groups would have been present, and we would have missed an interesting experiment.

The cleavage of the Boc groups was originally assumed to proceed without difficulties. However, various authors have reported that difficulties may occur. In our laboratory we have experienced that the cleavage of the Boc group from resin-bound phenylalanine with N HCl/HOAc was incomplete after 30 min at 20°, but complete at 25° [30].

Observations like this stress the importance of a quantitative controlling of every step in a solid-phase synthesis. Perchloric acid titration has also been used to monitor

syntheses carried out by other means of coupling than by DCC: thus, by condensation with o-nitro-phenylsulfenyl-protected N-carboxyanhydrides or activated esters, provided, of course, that the protection group does not allow protonation and is stable under the conditions of the titration procedure [31].

None of the methods used for a quantitative analysis on the total amount of resin-bound peptides are specific for amino groups. Thus, as mentioned above, the imidazole group of Boc-benzyl-histidine is also titrated, leading to an equivalent increase in the titration value [22].

The discussion of monitoring solid-phase syntheses will not be ended without mentioning the possibility of achieving this through a color change of the resin due to coupling and deprotection. Using quite another synthetic procedure than in the solid-phase peptide synthesis, namely by forming an active ester on the resin and letting this react with a carboxyl-protected amino acid or peptide, GUARNERI *et al.* obtained a color shift from yellow to green [32]. A prerequisite is that the reaction proceeds uniformly throughout the resin beads if the color change is to be measured by light reflection. If so, the principle may be applied in solid-phase synthesis, provided the use of a suitable colored protection group is feasible.

As previously mentioned, one of the interesting aspects of the solid-phase synthesis is the possibility of automation, due to the fact that the isolation of the resin-bound product can be carried out by simple filtration. We succeeded some years ago in developing a system in which the coding for the process was performed on punched tape [33, 34]. The control unit was based on sequential logics, which means that a return signal must be received making sure that a function has been carried out properly, before the next code can be read. Electronically, this is achieved through the use of electronic gates, here as integrated circuits. An alarm system paralyses the equipment if errors should occur in the function of the system.

It is possible to code for an entire synthesis. The system can initiate the function of analytical units as a titrator, but is incapable of evaluating analytical data, and consequently no change of a predetermined sequence of operations can take place automatically. The first prototype was ready in 1967, and the equipment marketed by Schwarz Bioresearch in 1969.

The control unit used in the original system is today outdated by a computer. The diagram shows the construction of the system at present in use in our institute, in which a minicomputer with an 8 K 16-bit core memory is used as a control unit (Fig. 5) [30]. As in the original system, the liquid is transported by nitrogen pressure in individual tubings, excluding the possibility of contamination.

The use of a computer as control unit has several advantages: a higher reliability of the electronics and flexibility for attachments of accessories such as analytical units, and an automatic evaluation of the analytical data leading to automatic decisions for the course of the synthesis. As an advantage it must also be considered that a complete print-out of the entire synthesis is obtained (Fig. 6). The coding for the synthesis is read via the teletype into the computer and checked for valid input. A letter indicates the proper function, and numeral the additional parameter. Thus, for example, in X2, the X means stirring, and 2 means for two minutes.

In the now further-developed system [35, 36] the titration is controlled by the computer. Automatic calculations are carried out on analytical data, and comparison is made with preceeding results. It is made possible by the programming to take a progressive change of value, such as a decrease due to cleavage of peptide from the

resin, into consideration in the comparisons. If more than one titration of a coupling or deblocking cycle is desired, the computer makes a comparison between consecutive titration values, and if a preset deviation is exceeded, the titration will be repeated until the last two obtained values are within the preset limit for deviation. To limit the number of titrations, if stability is not obtained, for example by increasing values due to cleavage of a protection group, the procedure is brought to an end after a preset number of titrations after a coupling or deblocking.

If the value for titration is accepted, and the comparison with the value after a previous coupling or deblocking is accepted, the synthesis will be continued according to the information on the punched tape. If the result of the titration leads to a repeating of the coupling or the deblocking cycle, this will be performed according to information stored in the computer during the last performed cycle. Thus, a stepwise feed-back system for process control is obtained through the described system (Fig. 7).

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VI  03568          START POTENTIAL (MV) :   +3.800000E+02
                   LAST POTENTIAL  (MV) :   +4.310000E+02
                   ADDED TITRANT   (ML) :   +1.300000E-01
                   MEQ                  :   +7.867600E-03
                   MEQ/G                :   +5.377606E-03
          00008    TERMINATED BY SHUT OFF TIME
YI  03576  00000
AI  03576  00001
X4  03577  00003
YI  03580  00000
AI  03580  00001
X4  03581  00003
YI  03584  00000
/2  03584  00000

#1  03584  00000
AI  03584  00001
X4  03585  00003
YI  03588  00000
AI  03588  00001
W0  03589  00001
AI  03590  00000
X4  03590  00003
X2  03593  00002
YI  03595  00000
AI  03595  00001
X4  03596  00003
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BI  03611  00001
X4  03612  00003
YI  03615  00000
BI  03615  00001
AI  03616  00000
VI  03616          START POTENTIAL (MV) :   +3.260000E+02
                   LAST POTENTIAL  (MV) :   +4.450000E+02
                   ADDED TITRANT   (ML) :   +1.900000E-01
                   MEQ                  :   +1.149880E-02
                   MEQ/G                :   +7.859578E-03
          00008    TERMINATED BY SHUT OFF TIME
                   TITR. ACCEPTED (MEQ) :   +9.683200E-03
                   COUPLING ACCEPTED
```

Fig. 6. Print-out from two titration cycles after removal of the Boc group. The three columns indicate: The code, total elapsed time, and duration of the single functions, both in minutes

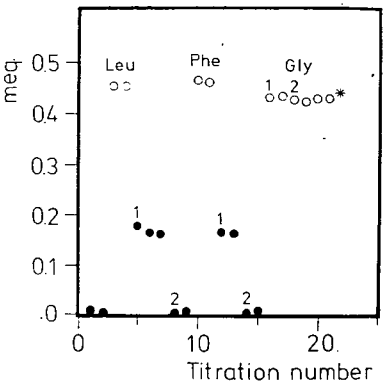

Fig. 7. Example of automatic decisions of the synthesizer. Preset value for deprotection: 0.470 (+0.015, −0.020) meq, allowing a slight decrease during the synthesis. Preset value for the coupling: 0.003±0.010. The last accepted mean value is used for comparison with the succeeding value for deprotection or coupling. Allowed deviation of titration values: 0.005 meq.

Only 75% of the theoretical amounts of Boc-amino acids and DCC are used leading to repetition of the coupling procedure for Boc-Leu and Boc-Phe. Too low value of deprotection of BocGly leads to a repetition of deprotection procedure. Finally, titration values are accepted, but too low mean value leads to interruption of the synthetic procedure. Two washings with methylene chloride, however, are performed to avoid damage until a new command is given to the synthesizer.

Filled circles before, and open circles after Boc-group cleavage. Paralysation.

3. Measurements on the liquid phase

However, it is desirable to have continuous information about the course of the single reactions. A principle for this, based on measurements of absorption in the ultraviolet region, was introduced by GUT and RUDINGER [37], and later used by BIRR [38], who developed an interesting reactor in which the liquid phase is circulated by centrifugation and monitoring is performed on the circulating fluid. The pathway of the flow cell is 0.5 mm.

Such a system provides the use of UV-absorbing protection groups and spectral stability of the dissolved components. The measurements must, if usable in practice, be performed at concentrations optimal for the synthesis. Such indirect measurements must always be considered with caution, as measurements on the liquid phase do not always exactly reflect what happens inside the resin. In spite of the mentioned reservations, such continuous non-destructive monitoring will certainly be of great value for optimizing solid-phase synthesis, as it will render information about the time-course of the single reactions and also about the distribution of the dissolved components between the interior of the resin and the sorrounding liquid, which may tell us about changes in the resin such as swelling.

To be able to operate at concentrations optimal for the synthesis, it is necessary in practice (due to the often high specific molar extinction of the amino acid derivatives) to use a flow cell with as small a light pathway as 0.03 mm. An experimental set-up with such a cell, using either a peristaltic pump or a reiprocating system, has been used for preliminary experiments. Further experiments must be performed to investigate whether the stepwise control by titration can be replaced by a continuous indirect method of monitoring on the circulating fluid. As mentioned above, however, we consider such a system as being of great interest.

Concluding remarks

The analytical methods at hand have proved valuable in the synthesis of shorter chains, especially by the solid-phase technique. It is to be expected that improvements of the analytical technique for both solid-phase synthesis and synthesis in solution will take place, hopefully also regarding control of racemization.

In the further investigation of the solid-phase technique, special attention has to be directed to a very important point, namely whether it is possible to improve the properties of the polymeric support.

The usually much longer coupling times in solid-phase synthesis of shorter peptides compared to synthesis in solution indicate that much could be gained through improvement. Hitherto, resins of the polystyrene type have been used almost exclusively, but other types of polymers have to be considered, such as polyacrylamide as proposed by ATHERTON and SHEPPARD [39].

In the synthetic reactions common to synthesis on a polymeric support and in solution, the fundamental problem has not been solved, namely the invention of a coupling reaction leading to 100% yield by a specific reaction between the α-amino group and the α-carboxyl group, thereby avoiding the use of covalently-bound protecting groups in the side-chains. As a matter of fact, the huge amount of work dedicated to invention of new protecting groups, purification and characterization procedures, has only been needed because no ideal coupling procedure is at hand. Will the problem be completely solved by imitation of nature, using components such as nucleic acids and enzymes? We do not know.

In Denmark we have a common phrase saying that it is difficult to prophesy — especially about the future — because it is no longer what is used to be. My personal opinion is that organic chemical synthesis of peptides in the forseeable future will be used for the synthesis of peptides of moderate chain length, and of course for the preparation of peptides with unusual structures, such as peptides labelled with isotopes in specific positions, or with a content of D-amino acids. However, for the synthesis of long peptide chains containing the usual protein-bound amino acids, quite another procedure may be the method of choice, namely genetic engineering, whereby genetic material is introduced into microorganisms, forcing them to synthesize special compounds. The increasing knowledge of the system for biosynthesis in the microorganism and the recent achievements in polynucleotide synthesis seem to justify great expectations for this technique. The synthesis of a complete gene for a tyrosine transfer RNA precursor from Escherichia coli by KHORANA and coworkers is certainly a strong impetus for the further exploitation of the possibilities for industrial production based on genetic engineering [40].

The reason for the rapid progress in the synthesis of polynucleotides is that in polynucleotide synthesis, in contrast to the synthesis of peptides, it is possible to combine non-protected fragments enzymatically in aqueous solution. The most important limiting factor in the synthesis of polynucleotides just now is the tedious preparation of the fragments, *i.e.* oligonucleotides. These are at present prepared by organic chemical synthesis in an organic medium. It would therefore mean a significant improvement, if an automated procedure were at hand. Various laboratories have investigated the possibility of carrying out the synthesis of oligonucleotides with the solid-phase technique, using the same type of resin ordinarily used in the synthesis of peptides, namely crosslinked polystyrene. The results however, have,

been negative. Recent experiments have nevertheless indicated that resin of the polyacrylamide-type are more advantageous [41]. Experiments carried out in our laboratory have confirmed this observation, as in the synthesis of pentathymidine-tetraphosphate it has been possible to obtain yields by the coupling of the single nucleotides of 84—90% [42]. We are therefore pursuing our efforts and hope that they will result in an automated synthesis of oligonucleotides based on the principles for automation already developed for the synthesis of peptides.

The initial studies of condensation of nucleotides to oligonucleotides were inspired by the achievements of the organic synthesis of peptides. It is indeed fascinating to realize that historically the experiment of CURTIUS in 1882 is thus linked to the modern understanding of the basic function of living matter.

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ЗАМЕЧАНИЯ ПО ПОВОДУ АНАЛИЗА СИНТЕТИЧЕСКНХ ПЕПТИДОВ

К. Брунфельдт

Существует три принципиальных возможностей для аналитического контроля эффективности степени связывания и освобождения функциональных групп в процессе твердофазного синтеза пептидов: 1) анализ образцов; 2) определение количества образовавшихся полипептидных связей; 3) измерения в жидкой фазе. Эти три разных метода обсуждаются подробно.