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A Computerized Peptide Synthesizer with Feed Back Control

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The feed back control in the automated system described is based on perchloric acid titration of free amino groups. If an unsatisfactory yield of coupling or deblocking is found, the coupling or deblocking may automatically be repeated. A complete print out of the synthesis is obtained. Two syntheses of leucineenkephalin are described, one with DCC in deficiency. The products are characterized by amino acid analysis, HPLC and mass spectrometry.

The system described is highly flexible regarding changes in the software as well as in the analytical, reactor and metering units. In principle, the basic construction of the synthesizer may be applied to solid phase synthesis of oligonucleotides.

Automatic monitoring was first achieved in 1972 by perchloric acid titration of free amino groups, allowing determination to be carried out, as well as following coupling and deblocking.[1] The method is not specific for α-amino groups as other protonable groups also are titrated, and the titration may be influenced by the structure of the peptide. Stable protecting groups under the conditions of the titration procedure are a prerequisite, and as in similar methods extra treatments of the resin-bound peptide are required. In spite of the above-mentioned, the method has shown itself to be valuable in the synthesis of medium-sized peptides due to the fact that quantitative measurements are performed of coupling as well as deblocking.[2]

Automatic monitoring by estimation of free amino groups was also achieved by photometric determination of liberated picric acid.[3] However, difficulties with the elution of picric acid with increasing peptide content seem to limit the usefulness of this method.[4]

Automatic monitoring based on UV absorption has also been published.[5] This principle is interesting, because continuous monitoring during the coupling as well as during removal of the α-amino protecting group is in principle possible. The method demands the use of a UV absorbing protecting group, and changes in the liquid phase may not exactly correspond to reactions on the polymer.[6] No doubt, however, valuable information may be obtained by the application of this principle, and the computerized synthesizer described here is therefore prepared to include monitoring based on UV absorption or a similar principle.

The present article describes a computerized peptide synthesizer with feed back control, based on perchloric acid titration of free amino groups after coupling and after removal of the α-amino protecting group. By automatic evaluation of the titration data by the computer, a decision is made whether the synthesis is to proceed or not. If the titration value indicates an unsatisfactory yield of a coupling or removal of the α-amino protecting group, the system can repeat automatically the coupling or the deprotection step. Thus, the computer controlled peptide synthesizer is able to run unattended as long as the course of the synthesis is satisfactory within preset parameters.

If optimum conditions for a synthesis have been determined, it may be advantageous to carry out a final synthesis with only a few steps monitored, thus limiting the extra number of treatments introduced by the titration procedure.

The two syntheses described in this article are given in order to elucidate the possibilities of the computerizer system.

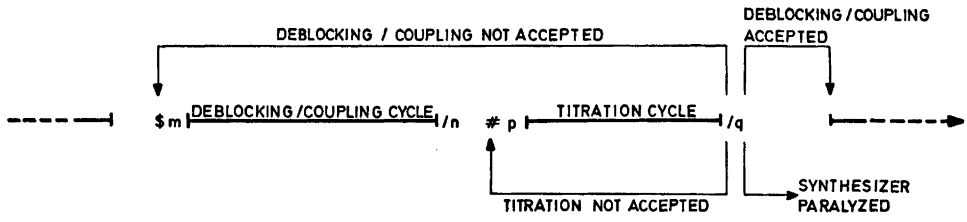

Fig. 1. Illustration of the feed back system.

SYSTEM DESCRIPTION

The computer-controlled peptide synthesizer using perchloric acid titrations for monitoring [7] has now been supplied with a feed back system, Fig. 1. In principle, the synthetic procedure consists of three cycles. A titration cycle, a cycle for removal of the α-amino protecting group (deblocking cycle), and a coupling cycle. However, the deblocking and coupling cycles are in principle handled in the same manner by the computer. A titration cycle starts with #p and is terminated by /q. A deblocking or coupling starts with $m and is terminated by /n. p and m are integers from 1 through 9, and q and n are integers from 0 through 9. p denotes how many times the titration cycle will be performed unconditionally, and q is the maximum number of permitted further titration cycles, in the attempt to obtain acceptable titration values. Similarly, m denotes how many unconditional deblocking or coupling cycles will be performed, and n is the maximum number of permitted further deblocking or coupling cycles that can be performed, if the titration value indicates an unsatisfactory deblocking or coupling.

When a start code is read from the tape, this code and the following codes will be stored in the computer memory, and the corresponding functions will be carried out. Storage of codes in the memory will stop, when the end code has been read and stored. This allows a repetition of a cycle without moving the tape. Thus at the end of the titration(s), the computer automatically evaluates the titration data resulting in one of four possibilities.

Table 1. Titration constants.

	Synthesis I Coupling		Deblocking		Synthesis II Coupling		Deblocking		
End points	EP1	425	EP2	425	EP1	425	EP2	425	mV
Shut off times	ST1	150	ST2	150	ST1	150	ST2	150	S
Proportional bands	PB1	−200	PB2	−10	PB1	−200	PB2	−10	mV
Proportional factors	PF1	0.04	PF2	0.03	PF1	0.04	PF2	0.03	
Start potentials, lower	SL1	200	SL2	200	SL1	200	SL2	200	mV
Start potentials, upper	SU1	550	SU2	400	SU1	550	SU2	400	mV
Initial milliliters	IM1	1	IM2	5	IM1	1	IM2	5	mL
Potential increases	PI1	10	PI2	10	PI1	10	PI2	10	mV
Max milliliters	MM1	1	MM2	10	MM1	1	MM2	10	mL
Addition factors	AF1	2	AF2	2	AF1	2	AF2	2	
Added milliliters	AM1	0.1	AM2	0.1	AM1	0.1	AM2	0.1	mL
Decimal factors	DF1	0.01	DF2	0.01	DF1	0.01	DF2	0.01	
Step numbers	SN1	7	SN2	7	SN1	7	SN2	7	
Grams of resin	GR1	1.53442	GR2	1.53442	GR1	1.53135	GR2	1.53135	G
Normalities	N1	0.0646	N2	0.0646	N1	0.06052	N2	0.06052	N
Titration deviations	TD1	0.005	TD2	0.005	TD1	0.005	TD2	0.005	meq
Titration alarm limits	TA1	−0.01	TA2	0.01	TA1	−0.01	TA2	0.015	meq
Titration repeat limits	TR1	0.005	TR2	−0.02	TR1	0.01	TR2	−0.02	meq
Titration values	TV1	0.003	TV2	0.47	TV1	0.003	TV2	0.47	meq

(1) The synthesis will continue if acceptable titration values and satisfactory deblocking or coupling yields are obtained.

(2) One more titration cycle will be carried out if permitted, in case acceptable titration values are not obtained.

(3) A repetition of the deblocking or coupling cycle and the titration cycle as indicated by p and q will be carried out if permitted, if reproducible titration value(s) are obtained but the deblocking or coupling yield are unsatisfactory.

(4) The synthesizer will be paralyzed if reproducible titration values or satisfactory values for deblocking or coupling cannot be achieved.

Titration. The titration equipment is controlled by the computer. The titration equipment consists of a high input impedance amplifier followed by an analog to digital converter and an autoburette. The titration equipment is connected to the computer *via* control interface for data and control signals transfer. This has resulted in advantages such as increased flexibility, check for proper electrode function, controlled limitation of added titrant, *etc.*

The code for titration is Vn, where n is an integer from 1 through 3, which allows three different titration circumstances. For instance V1 can be the code for titration after coupling, and V2 the code for a titration after deblocking allowing differentiation between titration values assigned to coupling or deblocking. Before starting a synthesis, the titration constants, Table 1, have to be loaded into the computer *via* the keyboard of the teletype.

The principle of the titration is shown in Fig. 2. When the code for titration has been read, the computer sets the respective constants and prepares the titration equipment. When done, a check of the start potential is carried out. If the start potential is not within the preset limits determined by *SL* and *SU*, indicating unsatisfactory electrode function, the synthesizer will be paralyzed after a preset number of steps *SN*, sufficient for a number of washings of the resin. An acceptable start potential results in a print out of the value. The computer now enters a titration loop, where it starts to check the terminating conditions. These are 'too slow addition of titrant' and 'shut-off time exceeded'. The first condition

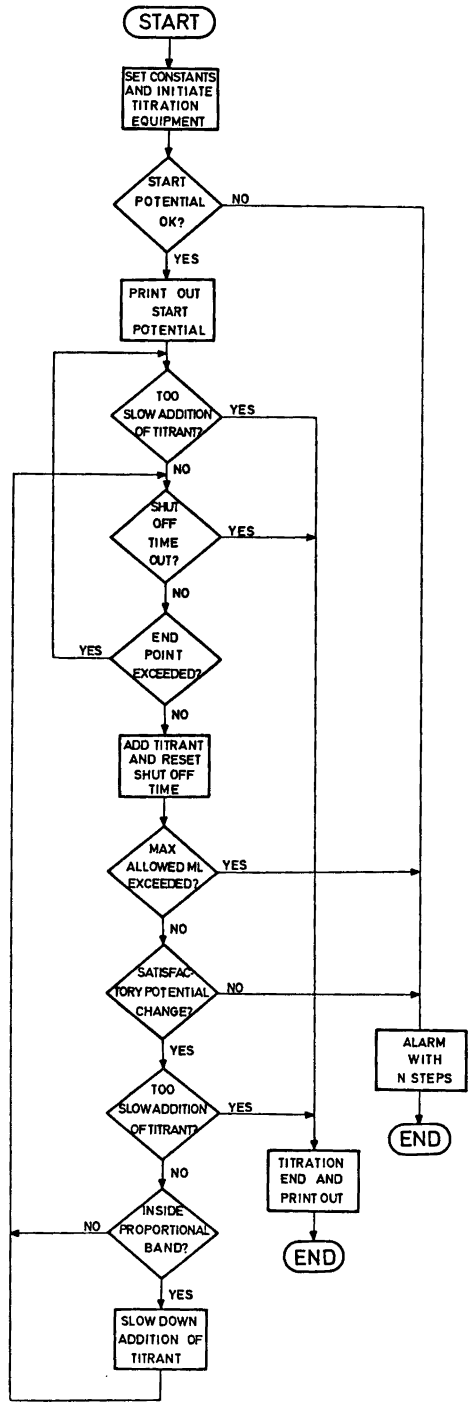

Fig. 2. Flow chart for the principle of the titration.

```
#2  03695  00000  START OF CYCLES
A1  03695  00001
X4  03696  00003
Y1  03699  00000
 |    |      |
 |    |      |
 |    |      |
 |    |      |
 |    |      |
B1  03722  00001
X4  03723  00003
Y1  03726  00000
B1  03726  00001
A1  03727  00000
V2  03727          START POTENTIAL [MV] :   +2.890000E+02
                   LAST POTENTIAL  [MV] :   +4.410000E+02
                   ADDED TITRANT   [ML] :   +7.840000E+00
                   MEQ                  :   +4.744768E-01
                   MEQ/G                :   +3.098421E-01
           00021   TERMINATED BY TOO SLOW ADDITION OF TITRANT
Y1  03748  00001
A1  03749  00000
X4  03749  00003
Y1  03752  00000
A1  03752  00001
X4  03753  00003
Y1  03756  00000
/2  03756  00000

#1  03756  00000
A1  03756  00001
X4  03757  00003
Y1  03760  00000
 |    |      |
 |    |      |
 |    |      |
 |    |      |
 |    |      |
B1  03787  00001
A1  03788  00000
V2  03788          START POTENTIAL [MV] :   +2.840000E+02
                   LAST POTENTIAL  [MV] :   +4.770000E+02
                   ADDED TITRANT   [ML] :   +7.800000E+00
                   MEQ                  :   +4.720560E-01
                   MEQ/G                :   +3.082613E-01
           00021   TERMINATED BY TOO SLOW ADDITION OF TITRANT
                   TITR. ACCEPTED [MEQ] :   +4.732664E-01
                   DEBLOCKING ACCEPTED
Y1  03809  00001
A1  03810  00000
X4  03811  00003
Y1  03814  00000
A1  03814  00001
X4  03815  00003
Y1  03818  00000
/2  03818  00000  END OF CYCLES
```

Fig. 3. Print out of two titration cycles after a deblocking cycle.

occurs if the addition of titrant during a period determined by the shut-off time ST multiplied by the additional factor AF is less than a preset amount of titrant AM.

Secondly, the titration is terminated if no titrant has been added during the shut-off time ST. When the titration is terminated, the values are printed out, Fig. 3. If the titration

is not terminated and the end point is exceeded, the computer will continue in the terminating loop. In case the end point is not exceeded, titrant is added. Each time titrant has been added, a check for maximum allowed amount of titrant *MM* is done. If *MM* is exceeded, the synthesizer will be paralyzed, as described above. This ensures that a malfunction in the system will not result in an unrestricted addition of titrant which can hurt the resin-bound peptide.

When a preset amount of titrant *IM* is added, the potential change must exceed a preset value *PI* to avoid a paralyzation of the system. This is done to ensure a satisfactory response from the electrodes. Now the condition for terminating the titration because of too slow addition of titrant is checked. In case of no termination, the computer examines whether or not the measured potential is within a preset proportional band *PB* just before the end point. Inside the proportional band the addition of titrant is reduced linearly, as the measured potential *E* approaches the end point *EP* according to eqn. (1).

$$X = \frac{ST \times PF}{PB} \times (EP + PB - E) \qquad (1)$$

where X is the delay between titrant-adding pulses and *PF* is a preset proportional factor, allowing a reasonable delay. Thereafter the titration continues with a check of whether or not the shut-off time is exceeded, until a terminating condition is obtained. This results in a print out of the last measured potential, added amount of titrant, total amount of milli equivalents, milli equivalents per gram resin, and the terminating condition, Fig. 3.

Feed back conditions. The feed back system is based on an evaluation of the obtained titration values, Fig. 4. As shown in Fig. 1, a titration cycle starts with #p and is terminated by /q. When p unconditional titration cycles have been performed, the deviation between the two last titration values is determined. If the deviation exceeds a preset value *TD*, the titration is not accepted and one further titration cycle will be carried out, if $q > 0$. When done, the deviation is determined as above, and if still not accepted, the titration cycle will be repeated, but only a maximum of q times. If acceptable titration values are not obtained after q repetitions, the synthesizer will be paralyzed. If the titration values are accepted, the computer determines the average value from the last two titrations, prints it out and stores it. In case of only one unconditional titration cycle, the value will always be accepted. By accepting the titration value after a deblocking, a comparison with the previously automatically accepted or manually set titration value after deblocking will be made, similarly for an accepted titration value after a coupling cycle.

For the deblocking as well as the coupling the last accepted titration value must be within

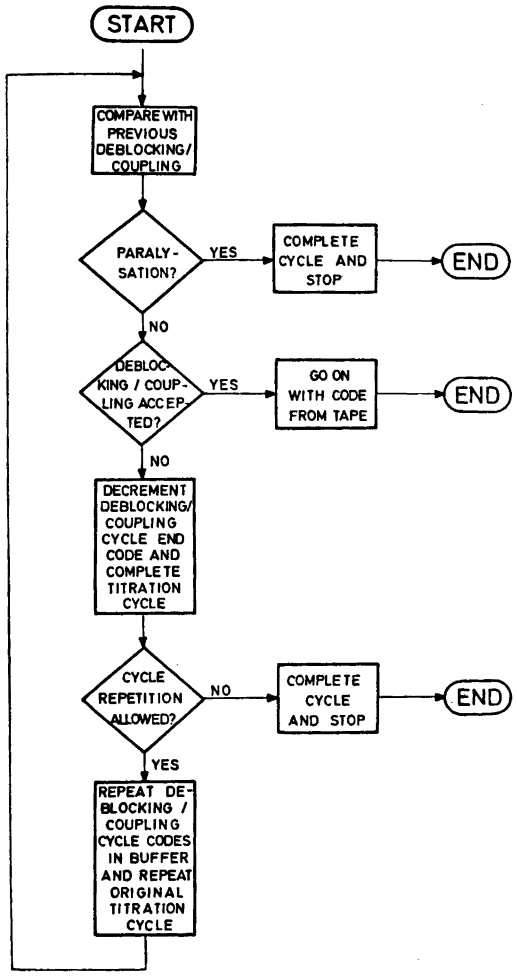

Fig. 4. Flow chart for titration cycle.

the limits determined by the previous accepted titration value *TV* and the limits *TR* and *TA*, if the synthesis is to continue without repeating the deblocking or coupling cycle. The deblocking cycle will be repeated provided $n > 0$, if the accepted average titration value is not higher than the lower limit determined by the previous titration value and *TR*, indicating insufficient deblocking. The syntheziser will be paralyzed, if the upper limit determined by the previous titration value and *TA* is exceeded. This is because an unexpected event has occurred, which will have to be considered by the operator. Such an irregularity could, for example, occur when an accumulation of incompletely removed protecting groups is, for one reason or another, cleaved in the just performed cycle. Similarly, the coupling will be repeated if the titration value is not lower than the upper limit, as determined by the previously accepted titration value and *TR*. The synthesizer will be paralyzed if the obtained titration value is lower than the lower limit determined by *TA* and the previously accepted titration value. In this case again an unexpected event has occurred which will have to be considered by the operator. Such an irregularity could for example be due to a complete coupling in the last performed cycle after an accepted progressive incompleteness of previous couplings. When a deblocking or coupling is accepted, the respective preceeding *TV* value is replaced by the respective new value. This allows a small decrease or increase in the yield of the next deblocking or coupling. In case of deblocking, the reason might be loss of α-amino groups due to irreversible blocking or loss of peptide. In the case of coupling the reason might be slightly insufficient couplings or side reactions.[8]

Manual operation. In order to increase the versatility, the synthesizer can also be operated manually. The synthesizer is transformed to manual mode *via* the teletype key board. The respective function is introduced *via* the keyboard using the usual syntax and the function will be executed with the normal controls.

When a tape has been prepared, it can be checked by the computer for valid characters and syntax errors before the synthesis is started.

MATERIALS AND METHODS

A Bio-Rad Beads S-X2 (200–400 mesh) resin was chloromethylated to 0.36 meq Cl g^{-1} chloromethylated resin.[1] Boc-Leu was esterified to the resin according to,[9] and a substitution degree of 0.34 mmol/g Boc-Leu-resin was obtained, determined by amino acid analysis.

Boc-amino acids were prepared according to Ref. 12, 2-BrBzl was used to protect the side chain of tyrosine.[11]

Titration is carried out as described in Ref. 17. The titration constants are shown in Table 1. A modified autoburette Radiometer ABU13 was set to 10 and a paper speed of 2 min cm^{-1} was used. As the amount of free amino groups after deblocking mostly decreases,[1,3,12] and the amount of titratable groups after coupling may increase, the titration constants were preset in order to allow this. In synthesis I, a decrease after deblocking of 0.02 meq (*ca.* 4 %) was accepted and for the coupling an increase of 0.005 meq (*ca.* 1 %). In synthesis II the corresponding values were 0.02 meq (*ca.* 4 %) and 0.01 meq (*ca.* 2 %). In both syntheses the deviation between two consecutive titration values were preset to a maximum of 0.005 meq (*ca.* 1 %). The cycles performed for coupling, deblocking and titration are shown in Table 2.

In synthesis I, DCC and Boc-amino acids were added in 4 times the theoretical amount. In synthesis II, DCC was added in 0.75 times the theoretical amount, and Boc-amino acids in 1.67 times the theoretical amount. Both syntheses were carried out with the reactor thermostated to 20 °C.

In order to achieve a check of the synthesis without introducing artefacts originating from cleavage of the product from the resin, amino acid analyses were performed on the resin-bound product [7] using a Beckman 121 or a Kontron Liquimat 3 amino acid analyzer. The peptide was cleaved from the resin by acidolysis with the resin suspended in a mixture of HOAc and CH_2Cl_2, 1/1 (v/v) with addition of 10 % 1,3-dimethoxybenzene and treatment with HBr for 5 h.[11] Using HOAc as acidic solvent to prevent formation of 3-(2-bromobenzyl)tyrosine, as previously demonstrated for preventing formation of 3-benzyltyrosine,[13] however, Tyr is *O*-acetylated. The crude peptide was neutralized with Et_3N and HOAc, dissolved in a small volume of DMF and precipitated with ether.

For removal of the *O*-acetyl groups on Tyr, samples of the ether precipitated products were dissolved in water, 1 M NaOH was added to pH 11.5 followed by stirring for 30 min at room temperature. 1 M HCl was then added to pH 6. After evaporation the remnant was dissolved in CH_3OH, filtered and precipitated with ether.

In order to evaluate the final products, HPLC was performed by an equipment from

Table 2. Cycles performed.

Number of treatments Titration	Deblocking	Coupling	Solvents and reagents	Vol/treatment ml	Time/treatment min
1	0	1	CH_2Cl_2	18	3
1	0	1	$CH_2Cl_2 + Et_3N + CH_2Cl_2$	18+1.5+18	5
2	0	6	CH_2Cl_2	18	3
2	1	0	HOAc	18	3
1	0	0	$HOAc + CH_2Cl_2$	18+18	titration
0	1	0	N HCl/HOAc	18	3
0	1	0	N HCl/HOAc	18	60 or 30 [a]
0	3	0	HOAc	18	3
0	0	1 (0) [b]	Boc-amino acid/CH_2Cl_2	18	0
0	0	1 (1) [b]	DCC/CH_2Cl_2	18	120 (0) [b]
0	0	0 (1) [b]	Boc-amino acid/CH_2Cl_2	18	120
2	0	2	CH_2Cl_2	18	3
0	0	4	EtOH	18	3
0	2	2	CH_2Cl_2	18	3

[a] In synthesis I, 60 min were used throughout the synthesis. In synthesis II, 60 min were used for deblocking of the C-terminal amino acid. In the succeeding deblockings 30 min were used. [b] When forming the tripeptide, DCC was added prior to the amino acid derivative.

Waters Associates with a μBondapak C_{18} column (30 cm length, 3.9 mm i.d.) run isocratically ($CH_3OH:H_2O:CH_3COOH = 50:50:1$) with a flow of 2 ml/min and monitored by UV at 254 nm.

Mass spectra were obtained on a Perkin-Elmer 270 mass spectrometer operating at 70 eV. The samples were introduced directly into the ion source and the temperature of the solids probe was increased from *ca.* 100 to 250 °C.

RESULTS

The titration values obtained during synthesis I of Tyr(2-BrBzl)-Gly-Gly-Phe-Leu-resin (leucin-enkephalin), using 4 times the theoretical amount of DCC and Boc-amino acids, is shown in Fig. 5. The titration cycle start code was $, and the end code was /2, which means that two unconditional titration cycles were carried out and two further titration cycles were allowed for obtaining acceptable titration values – for the set values, see Table 1. It is shown that it was necessary to carry out four titration cycles after the deblocking at the Leu-resin stage. The first titration value after the deblocking is seen to be lower than the succeeding ones, as often is the case. As the difference exceeds the set value, a further titration cycle is carried out. The lower first value may indicate that the applied procedure for removing the hydrochloride in the neutralisation step is unsatisfactory and that prolonged treatment or addition of a larger amount of triethylamine would be advantageous.

After the coupling, two titration cycles were sufficient for obtaining acceptable values.

The coupling cycle start code was $1 and the end code was /1, which means that one coupling cycle was carried out and one further cycle was allowed for obtaining acceptable coupling yield. The coupling with Boc-Gly to form a tetrapeptide resulted in an unacceptable yield, and the coupling cycle was therefore repeated. As the repetition did not result in

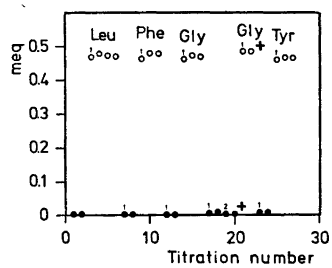

Fig. 5. Titration values determined during the synthesis of Tyr(2-BrBzl)-Gly-Gly-Phe-Leu-O-resin. ●, Titration value after coupling; O, Titration value after removal of the Boc group, deblocking; +, Paralyzation of the synthesizer. The indices denote the number of couplings or deprotections.

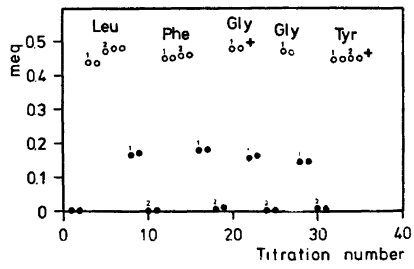

Fig. 6. Titration values determined during the synthesis of Tyr(2-BrBzl)-Gly-Gly-Phe-Leu-O-resin. ●, Titration value after coupling; O, Titration value after removal of the Boc group, deblocking; +, Paralyzation of the synthesizer. The indices denote the number of couplings or deprotections.

a better yield, the synthesizer consequently was paralyzed.

The deblocking cycle start code was $1 and the end code was /1. After the deblocking at the tetrapeptide stage, the synthesizer was paralyzed because the average titration value exceeded the upper limit, determined by *TA2* and the previous titration value. The value after deblocking of Tyr was accepted as a decrease was allowed.

The titration values obtained during synthesis II of Tyr(2-BrBzl)-Gly-Gly-Phe-Leu-resin, using 0.75 times the theoretical amount of DCC and 1.67 times the theoretical amount of Boc-amino acid, are shown in Fig. 6. The titration conditions are seen in Table 1, and the cycle start and end codes were the same as in synthesis I. As seen in Fig. 6 the coupling yields, as expected, were found to be unsatisfactorily low due to the use of less than equivalent amounts of DCC. After repetition of the coupling cycle, acceptable yields were obtained.

As also seen from Fig. 6, deblocking was repeated in three cases. The synthesis proceeded unattended to the tripeptide stage after deblocking, at which stage the synthesizer was paralyzed, because the average titration value exceeded the upper limit determined by TA2 and the previous titration value. Similarly, the synthesizer was paralyzed at the pentapeptide stage.

In Table 3 a comparison is shown between the amino acid content of the resin bound product determined by titrations and amino acid analysis. A reasonable agreement by the two analytical methods is demonstrated.

The difference in leucine content found by the two methods certainly is real, and may be explained by the difficulty of removal of the Boc-group of the ester-bound leucine.[4]

The slightly lower values determined for tyrosine in the peptides are due to partial destruction during the hydrolysis. In synthesis I the good agreement between the amino acid content determined by titration and by amino acid analysis of the crude cleaved product based on the total amount of amino acid per gram Boc-Leu-resin shows that the peptide in this

Table 3. Amino acid content determined by titration and amino acid analyses of the resin-bound product and the crude cleaved product. The amino acid content is determined as meq/g Boc-Leu-Resin. The number in brackets denote the relative content.

Amino acids	Titration	Amino acid analysis	
		Resin-bound product	Cleaved crude product
Synthesis I			
Leu	0.306 (0.99)	0.344 (1.12)	0.323 (1.02)
Phe	0.312 (1.01)	0.319 (1.04)	0.307 (0.92)
Gly	0.619 (2.00)	0.614 (2.00)	0.636 (2.00)
Tyr	0.300 (0.92)	0.274 (0.89)	0.303 (0.95)
Synthesis II			
Leu	0.316 (1.02)	0.340 (1.12)	0.292 (1.02)
Phe	0.304 (0.98)	0.329 (1.09)	0.282 (0.98)
Gly	0.618 (2.00)	0.605 (2.00)	0.524 (2.00)
Tyr	0.292 (0.94)	0.269 (0.89)	0.263 (0.92)

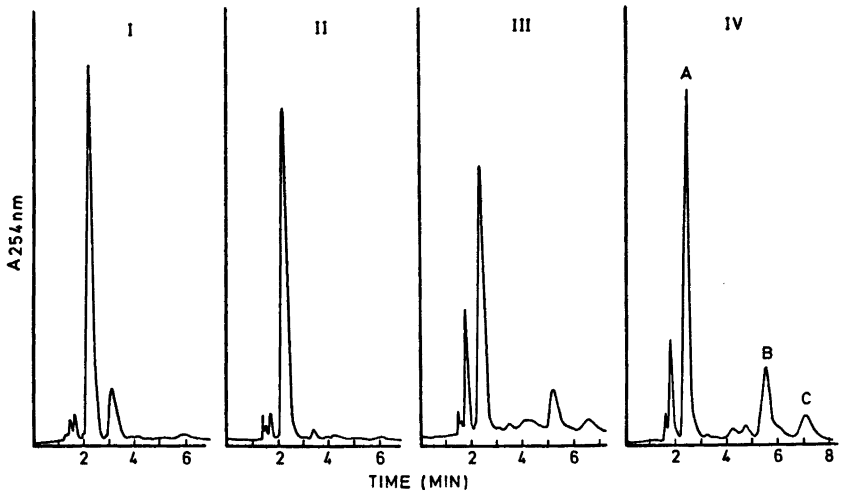

Fig. 7. HPLC chromatograms. I, Crude product from the synthesis carried out using 4 times the theoretical amount of Boc-amino acids and DCC. II, NaOH treated product from the synthesis carried out using 4 times the theoretical amount of Boc-amino acids and DCC. III, NaOH treated product from the synthesis carried out using 1.67 times the theoretical amount of Boc-amino acids, and 0.75 times the theoretical amount of DCC. IV, Preparative run of the product obtained as described in III. Samples I, II, and III: 50 μg, and IV: 1250 μg. The differences in retention time in III and IV are due to the amount of peptide.

case has been cleaved quantitatively from the resin. In synthesis II approximately 92 % of the peptide has been cleaved from the resin.

Comparing synthesis I and II, it is seen that it was necessary to repeat the deblocking in synthesis II at the *C*-terminal stage, although apparently identical circumstances were used. In synthesis I the synthesizer was paralyzed at the tetrapeptide stage after removal of the Boc-group and similarly in synthesis II at the tripeptide stage, because the upper limit was exceeded. This might indicate that unsatisfactory deblockings have been accepted at some earlier stage, which can give rise to deletion sequences as described in Ref. 14.

In Fig. 7, HPLC chromatograms of the two products are shown. It is seen that in synthesis I, a quite pure peptide is obtained after removal of the *O*-acetyl group on Tyr.

The chromatograms of the product from synthesis II after removal of the *O*-acetyl group on Tyr show that this product is more inhomogenous than the product obtained from synthesis I. A preparative separation was carried out, and the peaks A, B, and C, were collected. In 20 runs, 25 mg of the product dissolved in 2 ml of eluent were applicated and the peaks collected. The fractions were evaporated to dryness, redissolved in a few ml of HOAc and lyophilized. The obtained products were identified by amino acid analysis and by mass spectrometry after acetylation and permethylation.

Fraction A (*ca.* 9 mg) was identified as Tyr-Gly-Gly-Phe-Leu. Amino acid analysis, Tyr:Gly:Phe:Leu 0.95:2.03:1.01:1.00, the value for Tyr is uncorrected. The following two sequences were identified by mass spectrometry: CH_3CO-MeTyr-MeGly-MeGly-MePhe- and 4-$CH_3OC_6H_4CH$=CHCO-MeGly-MeGly-MePhe-MeLeu-OMe. The latter sequence is due to a fragmentation product from the tyrosine residue.

Fraction B (*ca.* 0.6 – 1 mg) was also identified as Tyr-Gly-Gly-Phe-Leu. Amino acid analysis, Tyr:Gly:Phe:Leu = 0.90:1.98:1.00:1.00; the value for Tyr uncorrected. The mass spectra were identical with the spectra obtained from fraction A.

Fraction C (*ca.* 0.2 mg) was identified to be a mixture of Tyr-Gly-Gly-Phe-Leu and Gly-Gly-Phe-Leu, the former in largest amounts.

Amino acid analysis, Tyr:Gly:Phe:Leu = 0.64:1.96:1.01:1.00; the value for Tyr uncorrected. A peak at the Asp position was present; however, no Asp could be detected by mass spectrometry. The following sequence could be identified by mass spectrometry: CH_3CO-MeGly-MeGly-MePhe-MeLeu-OMe as superposition to the spectra seen also in fraction A.

The standard deviation calculated from the values after deblocking as well as coupling, when two consecutive titrations have been accepted, has been determined according to $\sigma = \{[\sum(\Delta)^2]/2n\}^{\frac{1}{2}}$ where Δ is the difference between two accepted values and n the number of double determinations. The standard deviation found was 0.0016 meq, which equals 0.33 % based on the initial amount of amino groups. Supposing that the titration values for the coupling as well as for the deblocking are normally distributed, and that σ is known, a two-tailed u-test shows that a difference in yield of 0.8 % or greater can be detected at the 0.1 % level of significance, $\sigma \times U_{0.9995}/N^{\frac{1}{2}} = 0.33 \times 3.29/2^{\frac{1}{2}} \simeq 0.8$.

A statistical calculation for evaluation of identity of yields of consecutive cycles for deblocking or coupling is, however, of more theoretical interest than practical use. This because – with the present chemistry – the amount of free amino groups after deblocking often is found to decrease throughout a synthesis,[7,12,15–18] and also increasing values after coupling may be found.[1,3,19,20]

As mentioned under Material and Methods, unsymmetric preset limits of variation allow for the mentioned irregularities.

CONCLUSION AND DISCUSSION

The most significant advantage of the computerized system is the ability of the system to handle data from analytical units and thus be able to take decisions. The print out makes it easy to follow the procedure and facilitates the documentation.

The flexibility of the computer allows additional functions to be introduced by a change in the software. Thus alternative limits may be incorporated in the program and used consecutively if preset conditions for a synthesis cannot be fulfilled.

The analytical principle here applied is not to be considered ideal. As previously mentioned, its advantage is that quantitative measurements are obtained of coupling as well as deblocking.

Monitoring by light absorption involving the use of UV absorbing α-amino protecting groups is a highly interesting possibility, yielding continuous information. If, however, exact quantative measurements are to be achieved, the measured concentrations must be converted to total amounts, for example by the use of an internal standard.

The use of light absorption for monitoring is possible with the system described here, provided the development of the software and the proper interfacing.

The feed back principle of the computerized system is demonstrated by the two syntheses carried out. It is seen from the chromatogram of synthesis I, using Boc-amino acids and DCC in 4 times the theoretical amounts that the crude product is quite homogeneous and that purification using preparative HPLC can result in a pure product.

Synthesis II was carried out using only 75 % of the theoretical amount of DCC in order to establish conditions under which the synthesizer would decide to repeat the coupling cycle. It is seen that the major peak in the chromatogram has the same retention time as in synthesis I. It is confirmed by amino acid analysis and mass spectrometry that the main product has the expected composition Tyr-Gly-Gly-Phe-Leu. However, peak B and partly peak C, show peptides with the same sequence as in peak A. The explanation may be that under the unusual conditions of the synthesis one or more amino acid residues were converted to the D-form, or that other side reactions have taken place, undetected by the amino acid analysis and mass spectrometry, as the procedures for these two tests may lead to a cleavage of a formed derivative. In peak C a small amount of the shorter peptide Gly-Gly-Phe-Leu was also present, constituting 0.6 % of the total amount of the isolated product. The synthesizer as seen in Fig. 6 was paralyzed at the pentapeptide stage indicating a termination of glycine.

The computerized system is thus not only valuable for facilitating the synthetic procedure,

but may also be applied to the study of the reactions involved in the chain elongation.[21]

Recently, promising results were obtained by machine aided synthesis of oligonucleotides.[22] The basic principle of the synthesizer is also well suited to such a synthesis; however, changes of reactor and measuring system are necessary.

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