CONTINUOUS MONITORING IN SOLID PHASE PEPTIDE SYNTHESIS* **

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Continuous spectrophotometric monitoring on the recycled liquid in solid phase peptide synthesis is described. Rate measurements of some reactions are reported.

In solid phase peptide synthesis[2] difficulties have been encountered showing that experience gained from synthesis in solution cannot always be applied to the solid phase procedure[3-14]. Some complications may be circumvented by a proper choice of reaction conditions[12-20]. However, the very principle of building the entire peptide chain without intermediate purifications often leads to a final product of such complexity that analytical data may be of negligible value. Extraordinary high yields of the single steps in solid phase peptide synthesis are therefore imperative for achieving the desired product. As well qualitative[21,22] as quantitative [23-28] analytical procedures have been applied in order to estimate yields in the single steps. High accuracy was attained in automatic titration in non-aqueous media[29,30]. This method proved useful in manual as well as in automatic synthesis and divulged some factors which were responsible for low yields. However, determination of yields of the single steps will not reveal the course of the reactions. Information concerning the reaction dynamics must, therefore, be considered valuable for the optimization of single steps. A short account describing principle for achieving rate measurement in solid phase synthesis by continuous spectrophotometric monitoring has previously been published[1]. In the present paper a more detailed description is presented.

The basic principle for the apparative system used is that liquid phase is circulated and continuous spectrophotometric measurement is carried out on the circulating fluid (Fig. 1). The reaction vessel is made of glass and a sintered disc is inserted for the separation of resin and liquid. For agitation, a stream of nitrogen is introduced through the sintered glass disc in the reaction vessel. Splashing is minimized by conical shape of the vessel. The use of nitrogen ensures that reactions are carried out in an inert atmosphere. Before entering the reaction vessel nitrogen is saturated with solvent

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** For the preliminary communication see ref.[1].

vapours by passing a washing bottle equipped with a fritted disc as gas inlet. The washing bottle is kept at the same temperature as the reaction vessel. This treatment is necessary to maintain the constant volume of the reaction mixture during measurement. Adhesion of the polymer to the wall of the reaction vessel is reduced by silicon coating. Polymer adhering to the wall is washed down with solvent injected through an inlet in the lid. Reagents are introduced manually by removal of the lid. The liquid phase is removed by simultaneous suction and application of a nitrogen pressure of approximately 0·5 atm. The polymer is thus not exposed to the external atmosphere during the experiment. The liquid phase is circulated at a rate of 0·5–3 ml per min by means of a peristaltic pump and sucked from the reaction vessel through a filter. Teflon tubings were used for the connection of the reactions vessel to the flow-through cell of a single beam spectrophotometer. Measurements were carried out in UV or visible spectral region. Measurements in IR have been used for monitoring of organic chemical reactions[31] in a similar system.

In the case of coupling reactions in which a surplus of acylating reagent is used and a decrease corresponding to the amount coupled is measured, the measurement was performed at a relatively high absorbance, in order to achieve a higher percentage change of the signal with the decrease in concentration of the acylating agent. Measurement in the proper region was achieved by adjusting the optical path length of the flow-through cell. The path length required was calculated by $l = (\Delta A \,.\, E)/(\varepsilon \,.\, C)$, where l is the optical path length in centimeters, ΔA is the total absorbancy change, E is the reagent excess in multiples of the amino-acid amount or the peptide amount bound to the polymer, ε is the molar extinction coefficient and C the initial molar concentration of the reagent. For reactions monitored by the increase of a co-product concentration the path length was calculated by $l = (\Delta A \,.\, V)/(\varepsilon \,.\, Q)$, where

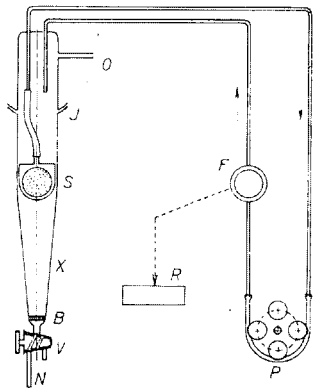

FIG. 1

Scheme of the Measuring Apparatus

X Reaction vessel, thermostating not shown; *B* porous glass disc bottom; *V* vacuum; *N* nitrogen; *J* spherical joint 35/25; *O* nitrogen outlet; *F* flow-through spectrophotometric cell; *R* recorder; *P* peristaltic pump.

Q is the number of millimoles of resin-bound amino acid or peptide, and V is the volume of reaction mixture in ml.

For the first type of experiments N-hydroxysuccinimide esters were used and *o*-nitrobenzenesulphenyl for amino group protection. The measurements were carried out at 384 nm. For the other type of experiments tert-butyloxycarbonyl-L-alanine 5-chloro-8-quinolyl ester was used. Measurements were carried out at 330 nm. Experiments comprising deprotection were performed using an *o*-nitrobenzensul-

TABLE I

The Coupling Rates of Activated Esters with a Phenalalanine Resin

Activated ester[a]	Conc.[b] M . 10^3	k, min^{-1}[c]	%[d]	Amino-acid ratio[e] (h)
Nps-Gly-ONSu	8	0·08	43—99	1·0 (24)
Nps-Ala-ONSu	8	0·038	51—99	0·90 (24)
Boc-Ala-OQ(Cl)	8	0·011	13—90	—
Nps-Pro-ONSu	8	0·0024	3—43	0·78 (96)
Nps-Pro-ONSu	40	0·0032	5—72	0·97 (25)
Nps-Pro-ONSu	80	0·0096	3—31	—
		0·0058	43—67	—
		0·0040	67—88	0·89 (24)
Nps-Val-ONSu	80	0·0024	11—70	0·90 (80)

[a] For abbreviations see ref.[33]; Q(Cl), 5-chloro-8-quinolyl; [b] in dimethylformamide, fivefold excess; [c] at 25°C; [d] procents of the completion of the reaction; [e] estimated by amino-acid analysis after splitting off from the resin.

TABLE II

Reaction Rates in Various Periods of Three Consecutive Peptide Couplings

Amino component on the resin[a]	k . 10^{-4}, min^{-1}			
	0—25%	25—50%	50—75%	75—90%
Phe	42	40	38	27
ValPhe	60	56	49	33
Val_2Phe	77	71	68	—

[a] 0·1M solution of *o*-nitrobenzenesulfenyl-L-valine N-hydroxysuccinimide ester in dimethylformamide as reagent in all stages (25°C).

phenylglycyl-L-phenylalanyl-resin. The cleavage of the protecting group was monitored at 384 nm.

The coupling rates of N-hydroxysuccinimide esters of some *o*-nitrobenzensulfenyl-amino acids are presented in Table I. The results indicate that the size of the side chain strongly influences the rate of the coupling reaction as is well known for syntheses in solution. The reported "first order" constants represent an approximation for the stated part of the reaction by the best fit of an exponential curve to experimental data. For the reaction course as a whole the deviation from first order kinetics was evident. When first order rate constants were calculated separately for the initial and successive stages of the same reaction, their values decreased with the advance of reaction (Table II). This effect is particularly pronounced in the reaction of the proline derivative (Table I). A similar decrease was also evidenced when the excess of reagent was taken into account. In this case, "second order" rate constants were calculated on basis of the decrease in concentration of the amino component as expressed by spectrophotometric measurements during the reactions, taking the decrease as a measure for the degree of coupling. The trend of decrease of the rate constant may indicate a heterogeneity in the reactivity of free amino groups in the polymer. Another explanation may be that the swelling of the resin was changing during the reaction.

A marked dependence of the reaction rate on the amino component has been observed in syntheses of two pentapeptides (Table III). In experiments in solution with activated phenyl esters, the structure of the amino component influenced the reaction rate[32-35] only to a slight degree. In syntheses shown in Table III the carboxyl-terminal amino acid affected reactivity of the valine amino group in the depeptide stage, thus indicating a marked sterical influence. This, however, may be an indirect effect due to a change in properties of the polymer. The reaction of tert-butyloxycarbonyl--L-alanine 5-chloro-8-quinolyl ester[37] shown in Table I was measured by the absorbance increase due to the resulting 5-chloro-8-hydroxyquinoline. A correction was made with respect to the observed liberation of the corresponding hydroxy derivative from the solution of the ester in dimethylformamide.

A dependence of the rate of cleavage of the *o*-nitrobenzenesulphenyl group with HCl on the composition of the reaction medium is shown in Table IV. The measurements were carried out at the isosbestic point at 284 nm in order to cancel the effect caused by the alcoholysis of the *o*-nitrobenzenesulphenyl chloride. In contrast to the coupling reactions, the cleavage of the *o*-nitrobenzenesulphenyl protecting group did not deviate from the first order kinetics. Whenever a 0·1M solution of *o*-nitrobenzenesulphenyl-L-valine N-hydroxysuccinimide ester in dimethylformamide was added to 1 g of resin, washed with ether and vacuum dried, a rapid decrease in transmittance was observed. That the increase in concentration was due to a selective uptake of solvent by the resin was demonstrated by the following experiment. After completion of the coupling reaction the product was freed from the surplus reagent by washing with ether and dried *in vacuo*. On addition to the solution of the activated ester a si-

milar decrease was observed. On the basis of increase in concentration of the activated ester and the swelling capacity of resin, the concentration of acylating agent in resin could be estimated. According to the calculation the concentration of acylating agent in polymer beads was found to be 0·045M, a value which differs significantly from the concentration in the added solution. The observed distribution phenomenon may markedly influence the velocity of the coupling reaction and thus be an explanation of the difference in the rate of reaction of the *ortho, meta* and *para* nitrophenyl esters in solution and solid phase peptide synthesis. Another explanation may be that steric hindrance in the resin hampers the reaction[38].

As described, two ways thus exist for monitoring the coupling reactions, *i.e.* measurement of coproduct formation or decrease in concentration of reactant. For both methods the stability of chromophore is a prerequisite. In both methods binding of the monitored compounds to the resin would influence the results. In case of the first method an instability of the activated ester would lead to erroneous result, whereas in the second case this would only happen if the cleavage of the ester bond

TABLE III

Half-Times of Reactions in Syntheses of Two Pentapeptides on a Resin

Sequence[a]	Ala—	Val—	Val—	Val—	Phe—	O—polymer
$t_{0.5}$ min[b]	0·5	98	124	173		
Sequence[a]	Ala—	Val—	Val—	Val—	Gly—	O—polymer
$t_{0.5}$ min[b]	0·5	91	91	17		

[a] Synthesized with the use of the *o*-nitrobenzenesulfenyl protecting group and activation in the form of N-hydroxysuccinimide esters at 25°C in 100 mM dimethylformamide solution, 0·04mm cell, measured at 380 nm; [b] the half-times were obtained directly from the graphical record of the reaction.

TABLE IV

Rates of Deprotection of *o*-Nitrobenzenesulfenylglycylphenylalanine Resin with 0·005M-HCl

Solvent mixture	k, min^{-1}[a]	%[b]
Dimethylformamide–methanol[c] 4 : 1	0·032	2—97
Dimethylformamide–2-propanol[c] 4 : 1	0·14	10—99

[a] At 25°C; [b] percents of completion of the reaction; [c] vol./vol.

would take place in a pronounced degree. In connection with automation using computer control, the monitoring described here can be applied in automated solid phase synthesis[39].

EXPERIMENTAL

Apparatus

The single-beam UV-visible recording spectrophotometer was assembled from parts manufactured by Zeiss-Jena and consisted of an SPM-1 monochromator, deuterium discharge lamp or a tungsten lamp, and an M 12 FQS 35 photomultiplier with a high voltage supply. A GIBI recorder recorded the transmission. The filter was made from two 0·2 mm distant sintered glass plates (grain size S2, Kavalier Glassworks, Czechoslovakia). The peristaltic pump was of the half-ring bed type, and the rate could be changed by change of the voltage. The action was reversed in experiments in which one gram or more of the polymer per 10 ml dimethylformamide was used. To prevent the filter from clogging, the action was reversed every 20—50 second for about 3 seconds. The reversal of the pump was synchronised with agitation by a stream of nitrogen passing through the porous bottom of the reaction vessel at a higher pressure than was normally used for agitation. On the recording, the reversals are seen as periodical deviations from the main course of the curve, and this does not interfere with the evaluation. The pump circuit was made from teflon tubings (i.d. 0·7 mm). Connections to the peristaltic pump, to the cell and to the reaction vessel were made from silicone rubber (i.d. 1 mm). The total volume of the pumping circuit was 1·6 ml. The pumping rate was adjusted according to the rate of the particular reaction in the range of 0·5 to 3 ml, mostly 1·5 to 2 ml per min.

Materials

As carrier was used chlormethylpolystyrene crosslinked with 2% of divinylbenzene (Calbiochem, U.S.A., Lot 801808; 5·47% Cl, 1·54 mmol/g.). Only derivatives of L-amino acids were used. The N-hydroxysuccinimide esters[40] of *o*-nitrobenzenesulphenylamino acids and the 5-chloro--8-hydroxyquinoline ester of tert-butyloxycarbonyl-L-alanine[41] were prepared by reported procedures. Dimethylformamide was dried over phosphorous pentoxide and distilled under reduced pressure.

Methods

Chlormethylpolystyrene was treated with tert-butyloxycarbonylglycine or tert-butyloxycarbonyl--L-phenylalanine as reported[42], except for the use of a closed reaction vessel. Mixing was performed by shaking with about 150 strokes per min for 24 h and maintained at 75°C by a thermostated jacket. The substitution of glycine and phenylalanine was 0·33 mmol/g and 0·39 mmol/g respectively. In reactions shown in Table I the protecting group was removed all at once for the whole amount of resin. In the reactions presented in Table II, the washings were performed as reported in the literature, omitting dichloromethane[42]. Liberation of the amino group from the hydrochloride was performed with triethylamine. In the experiments in which the amount of the solvent present in the polymer was not negligible compared to the total volume of the reaction mixture, the polymer after the triethylamine treatment was washed with four portions of ether and dried in the reaction vessel for 30 min at 15 Torr. During the measurement, the temperature

of the reaction mixture was maintained with an accuracy of ±0·1°C. The first-order rate constants were calculated from 25—50 time-transmittance data. The transmittance values were converted to absorbancy values, and these processed according to the literature[43]. Samples for amino-acid analyses were hydrolysed with a 1 : 1 vol./vol. mixture of 42% hydrobromic acid and fused phenol heated to 110°C for 24 h, filtered, and washed with three portions of glacial acetic acid and five portions of water and finally evaporated to dryness at about 1 Torr.

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