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# Kinetic Studies of the Liquid Phase Peptide Synthesis

**E. Bayer,* [1a] M. Mutter,[1a] R. Uhmann,[1a] J. Polster,[1b] and H. Mauser[1b]**

*Contribution from the Chemisches and Physikalisch-Chemisches Institut, Universität Tübingen, Tübingen, West Germany.* *Received March 4, 1974*

**Abstract:** Kinetic studies were carried out on the newly developed "liquid-phase" method (LPM) for peptide synthesis, using soluble polymer esters of amino acids. The reaction rates of these polymer-bound amino acids (esters of polyethylene glycol with molecular weights in the range of 2,000 to 20,000) were compared with those of the low molecular weight components used in classical peptide coupling reactions. New techniques for evaluating spectroscopic data have enabled the peptide coupling reaction to be studied precisely. The reactions were shown to be of the second-order type. The comparison indicated that the LPM system showed analogous kinetic behavior to the system used for classic peptide synthesis. The reaction rates are of the same order of magnitude and both systems revealed linear kinetic behavior. Under otherwise identical conditions the presence of the polyethylene glycol esters caused an increase in reaction rate compared with the corresponding low molecular weight analogs. This was interpreted as being due to tautomeric catalysis.

The "solid-phase" method for peptide synthesis, introduced some 10 years ago, was initially considered a very promising and exciting new technique which gave rise to great hopes and expectations.[2a] It thus seemed that the automated synthesis of biologically important peptides or even proteins was within reach.[2b-4]

However, criticism of the method grew as the new technique became popular in many laboratories.[5,6] In addition to pure synthetic problems the utilization of an insoluble matrix and therefore heterogeneous reaction conditions causes new difficulties such as steric hindrance,[7,8] solvation[9] problems, etc. These problems were of such importance that they tended to question the general viability of this technique.

In order to solve these problems, we have recently developed the "liquid-phase" synthesis which basically consists of the following:[10,11] a soluble, linear homopolymer serves as the C-terminal protecting group for the peptide which is to be synthesized. Mono- or bifunctional polymers such as polyethylene glycol have proved themselves as being especially good. All reactions are carried out under homogeneous conditions but contrary to the classical method, the activated components can be used in large excess so that quantitative coupling is achieved. The yields from the individual coupling steps can then be determined using simple tests.

Two properties of the polymer enable the polymer-bound peptide to be separated from the low molecular weight material, namely: (a) its molecular weight, which means separation can be achieved *via* ultrafiltration; (b) and the polymer's tendency to crystallize which remains unaltered by the peptide chain.[12,13] Thus, by using this new method the difficulties of the "solid-phase" synthesis can be avoided and at the same time its positive aspects are preserved. In this paper we wish to report the effect of the macromolecular protecting group upon the coupling reaction rate and compare the latter with that encountered in classical peptide synthesis, where low molecular weight protecting groups are used. In order to study the reaction rates, exact kinetic data were necessary.

If one succeeds in reaching rate constants using the "liquid-phase" method which are equal to those in classical peptide synthesis, no additional problems caused by the polymer would disturb a peptide synthesis. Then only the chemical problems of peptide coupling have to be considered.

The coupling reaction using *p*-nitrophenyl esters has proved itself to be especially suitable for kinetic studies since the reaction can be followed easily spectroscopically. In order to detect and recognize slight differences in the kinetic behavior of various substrates used here, it was found necessary to develop new and accurate techniques which have been mentioned elsewhere.[14] *tert*-Butoxycarbonylglycine *p*-nitrophenyl ester was coupled to various glycine esters under identical reaction conditions whereby the high and low molecular weight C-terminal protecting group was varied. The glycine esters of the following alcohols were used: *tert*-butyl alcohol, ethanol, 2-methoxyethanol, and polyethylene glycol with the molecular weight 2,000, 4,000, 6,000, 10,000, and 20,000. A comparison between polyethylene glycol triglycine ester and the triglycine ethyl ester should show whether the results obtained in synthesizing the dipeptide can be applied to larger peptides.

## Experimental Section

**Materials.** The following commercial products were used: acetonitrile (Uvasol Fluka and Uvasol Merck), further dried over molecular sieve (3 Å, Merck) and distilled; glycine *tert*-butyl ester hydrochloride (puriss, Fluka); glycine *tert*-butyl ester (puriss, Fluka); glycine ethyl ester hydrochloride (puriss, Fluka); triethylamine (puriss, Fluka); triglycine ethyl ester hydrochloride.[15] The commercial polyethylene glycols (Chemische Werke Hüls) were purified by ultrafiltration and by precipitation from solutions in methylene chloride with diethyl ether. The molecular weight distribution is less than ±7% of the molecular weights given.

**Synthesis of Glycine Polyethylene Glycol Ester Hydrochloride with Various Molecular Weights.** Polyethylene glycol (1 mmol) was dissolved in dry methylene chloride to form a 10% solution (w/v). BOC-Gly (1.75 g, 10 mmol) and DCC (2.06 g, 10 mmol) were added and the mixture was stirred at room temperature under anhydrous conditions for 6 days. The insoluble dicyclohexylurea was removed by filtration and the solvent removed *in vacuo*. The residue was then dissolved in 1.2 *N* HCl–acetic acid (10% solution, w/v) and stood at room temperature for 30 min to ensure complete cleavage of the protecting group.

The solution was evaporated to dryness and the residue dissolved in 150 ml of 10% sodium bicarbonate solution and this solution was ultrafiltered in a stirred cell (15 × 15 cm) with a Diaflo UM-2 membrane at 30 psi.[16] The solution volume in the cell was kept constant, replacing continuously the amount of filtrate penetrating the membrane by dilute bicarbonate solution. When no more amino acid was found in the filtrate after approximately 1-l. diafiltrate (3–6 hr) the solution in the cell was evaporated *in vacuo* to dryness and the residue dried by repeated azeotropic distillations using benzene. In the case of polyethylene glycol ester with molecular weight 2000, the excess of amino component was removed by using Sephadex chromatography (G-25) instead of ultrafiltration. The degree of esterification was quantitatively determined by amino acid analysis after acid hydrolysis and the following values were obtained: PEG (20,000), 0.0828 mmol/g ≅ 83%; PEG (10,000), 0.158 mmol/g ≅ 79%; PEG (6000), 0.266 mmol/g ≅ 79%; PEG (4000), 0.38 mmol/g ≅ 76%; PEG (2000), 0.488 mmol/g ≅ 49%.

**Preparation of Glycine 2-Methoxyethyl Ester Hydrochloride.** Glycine (10 g) was added to freshly distilled methyl cellosolve (100 ml) and the suspension was saturated with hydrogen chloride gas. The mixture was then cooled in an ice bath and resaturated with hydrogen chloride gas. The solvent was removed *in vacuo*, leaving an oil which crystallized on treatment with an ether–dioxane mixture and was recrystallized from ethanol–dioxane–ether as needles. Further recrystallization from warm chloroform yielded 14.9 g (66%), mp 107–108°. Calcd: C, 35.40; H, 7.13; N, 8.26; Cl, 20.90. Found: C, 35.76; H, 7.05; N, 8.13; Cl, 21.12.

**Synthesis of H-Gly-Gly-Gly-O-PEG · HCl.** The synthesis followed the general procedures described in ref 11, 16b, 17. H-Gly-PEG · HCl (mol wt 20,000; 2 g, 0.038 mmol/g) was dissolved in methylene chloride (20 ml) to which *N*-methylmorpholine (0.08 mmol) was added. BOC-Gly (0.15 mmol) and DCC (0.15 mmol) were added to the clear solution which was stirred at room temperature for 30 min. The dicyclohexylurea was removed by filtration and the solvent removed *in vacuo*. At this time a quantitative ninhydrin test was performed on a small sample which had been purified from excess amino acids by ultrafiltration. The BOC group was removed by treatment with 1.2 *N* HCl–acetic acid for 15 min at room temperature and the solvent removed *in vacuo*. The residue was taken up in water, the pH adjusted to 6, and ultrafiltered by the procedure described above.[16b] The water of the aqueous solution retained in the ultrafiltration cell was then removed by freeze drying. The tripeptide H-Gly-Gly-Gly-O-PEG · HCl was synthesized in an analogous way using H-Gly-Gly-O-PEG ·HCl. The yield of tripeptide polymer was 1.95 g. In order to check the purity the peptide was cleaved from the polymer using a small sample of tripeptide polymer by treatment with 1 *N* potassium hydroxide for 10 min. After neutralization the mixture was chromatographed on Sephadex G-25 and the ninhydrin positive fraction was pooled. The purity of this product was checked by thin layer chromatography, elementary analysis, electrophoresis, and especially by high-pressure liquid chromatography of the dimethylnaphthylsulfonyl derivatives: column 50 × 0.3 cm, silica gel 5 μ; eluent benzene:pyridine:acetic acid:2-propanol 50:50:5:5 (v/v); flow rate 1 ml/min, 210 atm. Only triglycine and no glycine or diglycine could be detected.

**Evaluation of Kinetic Data.**[18] The "EDTQ" program and the "formal-integration" methods[19] were used to determine the rate constants. The theoretical treatment of these methods has recently been published.[14]

For the "EDTQ" program where the starting concentrations of A and B have to be equal (reaction A + B → C + D), the following equation is of importance

$$(E_l - E_{0l}')/(t - t') = kaE_{\infty l} - kaE_l \quad (1)$$

where $E_l$ = extinction at time $t$ at wavelength $l$, $E_{0l}'$ = extinction (1.2 or $n$th value as reference) at time $t'$ at wavelength $l$, $E_{\infty l}$ = extinction at time $t = \infty$ at wavelength $l$, $k$ = second-order rate constant, $a$ = concentration for substance A at time $t'$ (when $t'$ = 0, than $a = a_0$). Using the "formal-integration" method eq 2 should also be considered

$$(\ln a/a_0)/t = k(a_0 - b_0) - k\left(\int_{t=0}^{t} a\,dt\right)\Big/t \quad (2)$$

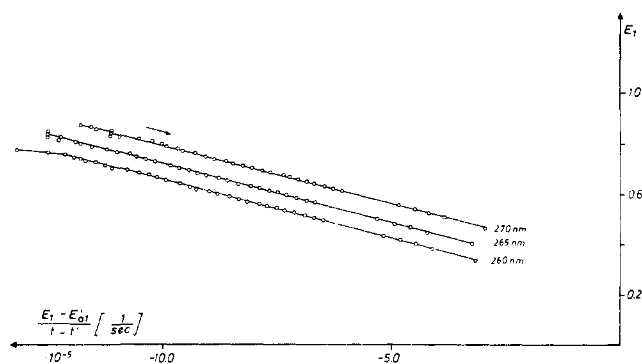


**Figure 1.** The $E_l$-$(E_l - E_{0l}')/(t - t')$ diagram (270, 265, 260 nm) for the reaction between BOC-Gly-ONP, *tert*-butylglycine ester hydrochloride, and triethylamine in acetonitrile (concentration of each component: 9 × $10^{-4}$ *M*).

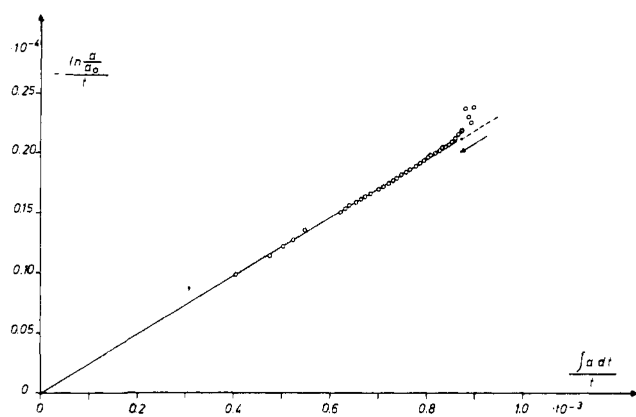


**Figure 2.** The diagram derived from eq 2 for the reaction between BOC-Gly-ONP, *tert*-butylglycine ester hydrochloride, and triethylamine under the same reaction conditions as for Figure 1.

where $a_0$ and $b_0$ = initial concentration of substance A or B, $a$ = concentration of A at time $t$, $k$ = second-order rate constant ($mol^{-1}$ $sec^{-1}$). The constant $k$ can be calculated from the gradients of the corresponding diagrams. In the $E_l$-$(E_l - E_{0l}')/(t - t')$ diagrams, one obtains a series of parallel lines for the various wavelengths (Figure 1) but only under the condition that the initial concentrations of A and B are identical ($a_0 = b_0$). The reaction proceeds in the direction of the $E_l$ axis. The point of interception on this axis gives the extinction when the reaction has gone to completion (100%, $E_{\infty l}$). The gradient of the $E_l$-$(E_l - E_{0l}')/(t - t')$ diagrams increases steadily when one takes as reference for $E_{0l}'$ the first, second, or $n$th extinction at constant wavelength $l$. The straight lines intercept at $E_{\infty l}$. The second-order rate constants can be calculated from the gradients according to eq 1 and the values measured at various wavelengths. The concentration of A at time $t$ can be determined using the "$E$ diagram"[20] which can be transformed to Figure 2 using eq 2. The rate constant can be determined independently from the gradient in Figure 2. The spectra were measured using a recording spectrometer DMR 21 WL (Zeiss) equipped with thermostated cell holders. The temperature was maintained at 25.0 ± 0.2° with a Haake thermostat 70. The measurements using more than one wavelength were carried out on a Zeiss spectrometer PMQ II with a double monochromator MM 12, automatic slit regulator, and thermostated cells. A deuterium high-pressure lamp (Zeiss) was used and the reactions were performed in 1-mm quartz cells (Hellma, Nr. 110 QS).

The extinction values at 320, 310, 270, 265, 260, and 230 nm were measured repetitively and the values with the corresponding reaction time were recorded on computer punch tape. The kinetics was followed continuously[21] contrary to the normal methods used in peptide chemistry up till now.[22-24] The kinetic data were analyzed using a CDC 3300 computer (Control Data Co.) with the following programs (FORTRAN): DIGIMESS, SYNCHRO, KINPLOT 3, EDTQ 2, FORMALIN, and KINALYSE.[14]

The reaction was started thus: the acetonitrile solution (3 ml)

**Table I.** Rate Constants for the Coupling Reaction BOC-Gly-ONP + $(Gly)_n$-Ester in Acetonitrile, Evaluated According to the "EDTQ" Program (eq 1) and the "Formal-Integration" Method (eq 2) (Reaction Temperature 25.0 ± 0.2°)

| Reaction: BOC-Gly-ONP + amine component | Rate constant $k$ (mol$^{-1}$ sec$^{-1}$) according to Eq 1 | Eq 2 | Molar concentration of The amine component | TEA | Reaction followed to $n$% completion where $n$ = |
|---|---|---|---|---|---|
| Gly-OBut·HCl | $0.24 \times 10^{-1}$ | $0.26 \times 10^{-1}$ | $9 \times 10^{-4}$ | $9 \times 10^{-4}$ | 75 |
| Gly-OBut | $0.23 \times 10^{-1}$ | $0.24 \times 10^{-1}$ | $9 \times 10^{-4}$ | | 78 |
| Gly-OEt·HCl | $0.12 \times 10^{-1}$ | $0.13 \times 10^{-1}$ | $10^{-3}$ | $1.25 \times 10^{-3}$ | 80 |
| $(Gly)_3$-OEt·HCl | $0.19 \times 10^{-1}$ | $0.19 \times 10^{-1}$ | $10^{-3}$ | $1.05 \times 10^{-3}$ | 52 |
| Gly-2-Methoxyethyl Ester·HCl | $0.71 \times 10^{-2}$ | $0.79 \times 10^{-2}$ | $10^{-3}$ | $1.05 \times 10^{-3}$ | 42 |
| Gly-O-PEG(20,000)·HCl | $0.12 \times 10^{-1}$ | $0.14 \times 10^{-1}$ | $10^{-3}$ | $1.05 \times 10^{-3}$ | 57 |
| Gly-O-PEG(10,000)·HCl | $0.12 \times 10^{-1}$ | $0.14 \times 10^{-1}$ | $10^{-3}$ | $1.05 \times 10^{-3}$ | 60 |
| Gly-O-PEG(6,000)·HCl | $0.13 \times 10^{-1}$ | $0.16 \times 10^{-1}$ | $10^{-3}$ | $1.05 \times 10^{-3}$ | 64 |
| Gly-O-PEG(4,000)·HCl | $0.17 \times 10^{-1}$ | $0.19 \times 10^{-1}$ | $10^{-3}$ | $1.05 \times 10^{-3}$ | 75 |
| Gly-O-PEG(2,000)·HCl | $0.16 \times 10^{-1}$ | $0.18 \times 10^{-1}$ | $10^{-3}$ | $1.05 \times 10^{-2}$ | 65 |
| $(Gly)_3$-O-PEG(20,000)·HCl | $0.32 \times 10^{-1}$ | $0.31 \times 10^{-1}$ | $10^{-3}$ | $1.05 \times 10^{-3}$ | 86 |

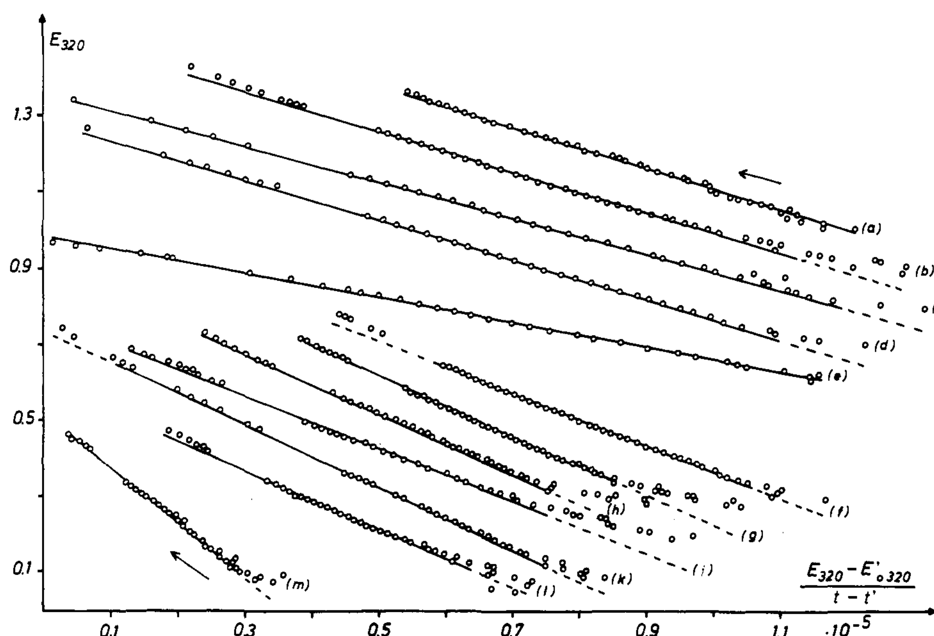


Figure 3. Combined $E_l$-$(E_l - E_{0l}')/(t - t')$ diagram for all reactions studied (320 nm). The origin of the coordinates for each curve have been shifted according to the values ($x/y$) given in parentheses where $(E_{320} - E_{0,320}')/(t - t') = x$ axis and $E_{320} = y$ axis: (a) $(Gly)_3$-ethyl ester·HCl (−0.1/+0.9), (b) Gly-PEG(4000)·HCl (−0.1/+0.8), (c) Gly-OBut·HCl (−0.35/+0.7), (d) Gly-OBut (−0.3/+0.6), (e) $(Gly)_3$-PEG(20,000)·HCl (−0.15/+0.3), (f) Gly-PEG(2000)·HCl (0/+0.2), (g) Gly-PEG(10,000)·HCl (0/+0.2), (h) Gly-PEG(6000)·HCl (−0.1/+0.2), (i) Gly-OEt·HCl where TEA = $5.10^{-3}$ $M$ (−0.25/+0.1), (k) Gly-OEt·HCl where TEA = $1.25 \times 10^{-3}$ $M$ (−0.2/0), (l) Gly-PEG(20,000)·HCl (−0.2/−0.05), (m) Gly-2-methoxyethyl ester·Cl (−0.35/0).

containing the corresponding amino component (for concentration see Table I, column 3) was thoroughly mixed with a stock solution of BOC-Gly-ONP in acetonitrile (0.03 ml; concentration = 100 × concentration of the amino component) and a standard TEA solution in acetonitrile (0.03 ml; 100 × concentration, given in Table I, column 4). An aliquot of the mixture was immediately transferred to the 1-mm quartz cell and the stop watch started.

## Results

**Aminolysis of *tert*-Butoxycarbonylglycine *p*-Nitrophenyl Ester by Glycine Esters of Low Molecular Weight Alcohols (Classical Coupling).** The reaction rate of *tert*-butyl ester of glycine was initially determined, because of this ester being most frequently used as the *C*-terminal protecting group for classical peptide synthesis. The second-order velocity constant $k$ has a value of $0.25 \times 10^{-1}$ mol$^{-1}$ sec$^{-1}$ (Table I) and is approximately 100-fold smaller than the rate of aminolysis using *n*-butylamine ($k$ = 2.78 mol$^{-1}$ sec$^{-1}$).[14] This result can be explained by the lower nucleophilicity of amino acid esters compared with primary amines.

Due to its high reaction rate, *n*-butylamine can be used to destroy the excess *p*-nitrophenyl ester at the end of the coupling reaction. No difference in the velocity constants could be detected compared with the free base when the hydrochloride of the *tert*-butyl ester was used and the reaction started by addition of a stoichiometric quantity of triethylamine. The ethyl ester of glycine reacts approximately half as fast as the *tert*-butyl ester (see Table I).

Glycine 2-methoxyethyl ester is the simplest low molecular weight model compound for the esters of the polymer, polyethylene glycol. Hence this compound was also investigated kinetically, and the coupling reaction was followed up till 40% completion. The slower reaction rate can be well explained by the stronger $I$ effect of the ester group, compared with the ethyl ester. The $k$ value for triglycine ethyl ester lies between those of the *tert*-butyl and ethyl esters. One would expect a higher reaction velocity due to electronic effects, but these results show that obviously steric factors play a more important role. Surprisingly, the reaction rate is small compared with that of the polymer-bound tripeptide; this fact will be discussed later.

**Aminolysis of *tert*-Butoxycarbonylglycine *p*-Nitrophenyl Esters by Glycine Esters of Polyethylene Glycol with Varying Molecular Weight of the C-Terminal Protecting Group ("Liquid-Phase" Coupling).** Various polyethylene glycol esters with molecular weights of 20,000, 10,000, 6,000, 4,000, and 2,000 were investigated in order to determine the effect of the molecular weight of the amino component upon the reaction rate. As seen in Table I, the reaction velocity increases with decreasing molecular weight. A noticeable jump is obvious between the molecular weights 6000 and 4000, whereas no further change occurs in going from a molecular weight of 4000 to 2000.

The slight decrease in the $k$ values with increasing molecular weight closely parallels the increase in the viscosity of the solution. The most important result of this study is that the rates of aminolysis of the macromolecular glycine esters in the "liquid-phase" system lie in the same order of magnitude as that of the ethyl ester of glycine. In addition to this, the low molecular weight analog of the polyethylene glycol ester, namely the 2-methoxyethyl ester, couples only half as rapidly to the dipeptide as the corresponding macromolecular ester. The same tendency can be observed when comparing the two tripeptides in the reaction to the tetrapeptide. The ethyl ester of triglycine achieves only two-thirds of the reaction velocity of the triglycyl polyethylene glycol ester and in spite of the large molecular weight of the latter, namely 20,000, it shows the highest reaction rate of all esters studied.

## Discussion

Figure 3 shows the curves derived from eq 1 for the various reactions measured at the same wavelength (320 nm); the values for all the reactions approach the $E_l$ axis. A flat curve denotes a high reaction rate. According to theory, straight lines should be obtained for the reaction $A + B \rightarrow C + D$. As can be seen straight lines are obtained after the initial phase of the reaction. Only in this initial phase the reaction rate is higher in comparison with the linear range both for classical and the "liquid-phase" coupling.

Theoretically, there are many potential sources for such slight deviations from linearity. Apart from inaccuracies in measuring which would be most noticeable in the initial stages of the reactions, side reactions are possible, such as the formation of diketopiperazine and hydrolysis, due to traces of water in the acetonitrile used.

For the coupling of the polymer esters the molecular weight range of the polymer may affect the linearity of the reactions. In order to obtain reliable rate constants, these were always calculated from the linear parts of the curves. The ratio of the rate constants for the glycine polyethylene glycol ester (20,000) and glycine 2-methoxyethyl ester is 1.74 and is very close to that for the triglycine polyethylene glycol ester (20,000) and triglycine ethyl ester, namely 1.66. One should expect very similar values due to comparable electronic environments at the reaction centers but the fact that these values are larger than 1.00 is important and significant. Thus, the polymer reaction proceeds at a faster rate than the reaction involving low molecular weight components (average factor: 1.70). Apparently the polyether acts as a catalyst for the reaction similar to those tautomeric catalysts like benzoic acid, 2-pyridone, pyrazole, imidazole, 1,2,4-triazole, etc.[25,26] In their kinetic studies on the "solid-phase" system Gut and Rudinger[22] observed a considerable drop in the reaction rate once the coupling had passed about 50% completion, as did Andreatta and Rink[24] in their investigations on a soluble, polyfunctional polystyrene polymer. The reason for this nonlinear kinetic behavior in these cases was thought to be due to the presence of reactive centers with differing reactivity. Such behavior was not noticed in the "liquid-phase" system although the individual reactions were followed up to 60–80% completion (see Table I).

Consequently, when used as a support in peptide synthesis polyethylene glycol shows homogeneous kinetic behavior and the rate constants obtained are equal to those found for classical coupling reactions and in certain cases superior. In conclusion it can be said that these kinetic studies have confirmed the very favorable results obtained so far for the "liquid-phase" method for peptide synthesis. Thus this combines the positive aspects of the already existing techniques, namely (a) the inherent simplicity of synthesis using macromolecular supports and (b) the obvious advantages of carrying out the peptide synthesis under homogeneous and kinetically optimal conditions.

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