# A PROBLEM IN THE MECHANISM OF CARBODIIMIDE-MEDIATED SYNTHESIS OF PEPTIDES IN AQUEOUS MEDIUM[1]

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Received April 19, 1973

SUMMARY

Leucylleucine was synthesized in aqueous solution using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide as the condensing agent. The yield was strongly dependent on acid concentration and was maximal at about 0.1 M HCl. The nature of this dependence suggests that the mechanism established for aprotic systems may not apply at all acid concentrations to aqueous peptide syntheses.

## INTRODUCTION

Carbodiimides are widely used (1) as condensing agents in peptide synthesis. Therefore, thorough understanding of the process is important. The mechanism proposed (2) for this synthesis is shown in Figure 1. It has been confirmed (3) for aprotic solvents and suggests that the last step is nucleophilic displacement by a basic amino group. Hence, an amino acid taking the carboxylate-terminal position of the peptide should react only when its amino group is unprotonated. This requirement conflicts with the initial need to protonate both carbodiimide and postulated O-acyl urea. As a result, yield of dipeptide should rise to a maximum and fall quickly to zero when acid concenration is high enough to protonate carbodiimide, O-acyl urea and amino group.

In the pH range between $pK_a$ values of carboxyl and amino groups, there is (4) a small fraction of uncharged isomer in equilibrium with zwitterion form. Therefore, peptides could form in aqueous systems at pH values above

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[1] Authorized for publication as paper no. 4160 in the journal series of the Pennsylvania Agricultural Experiment Station.

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$pK_{a_1}$ of the amino acids concerned. Below this pH by a unit or more, the amino acid is completely protonated (4), free α-amino group cannot be present to act as a nucleophile, and peptide should not be formed. During work (5) on selectivity in aqueous peptide synthesis, this prediction was tested by studying yield of leucylleucine as a function of HCl concentration.

## METHODS

Equal volumes of 0.06 M leucine in HCl and 0.100 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride in water were mixed. HCl concentrations of the leucine solutions were chosen to make their contributions to the final reaction mixtures 0, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, and 1.0 M. Reaction systems were held at room temperature overnight. Afterward, 10 µl of each was fractionated by thin layer chromatography using leucylleucine as a standard. Plates were 20 x 20 cm, 0.5 mm silica gel G, developed at room temperature for 4 hours, one set in 1-butanol/acetic acid/water (80:20:20 v/v/v) and one set in t-butanol/2-butanone/water/ammonium hydroxide (40:30:20:10 v/v/v/v). They were air dried, sprayed with 0.4% ninhydrin in ethanol and heated for 2 minutes at 110° C. Components chromatographing as leucylleucine in both solvents were measured with a Photovolt Multiplier Photometer 520-A using a thin layer plate stage attachment and a Varicord 433 recorder. Yields of leucylleucine were read from recorder charts and normalized by comparison with the value at 0.1 M HCl, the concentration producing the highest yield.

## RESULTS AND DISCUSSION

Figure 2 shows results from one experiment but is typical of four individual trials. Low HCl concentration reveals a catalytic effect by acid. At concentrations of 0 and 0.01 M, no dipeptide formed although two other products were revealed by chromatographic analysis. Both contained leucine and condensing agent. One of these products decreased steadily with increasing HCl concentration; the other disappeared when leucylleucine was formed (0.05 M HCl).

Although the general mechanism of Figure 1 refers only to synthesis of peptides, other products may be formed by side reactions, probably as follows.

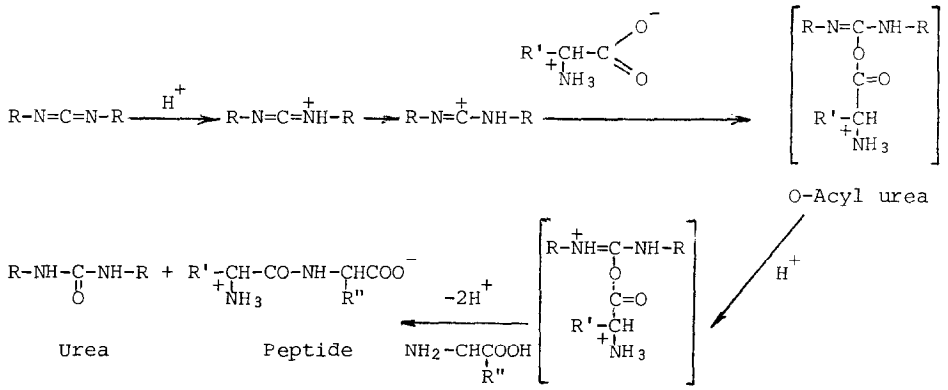
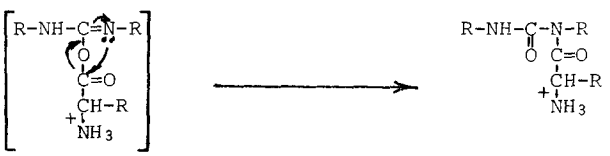



Fig. 1. A mechanism proposed (2) for carbodiimide-mediated peptide synthesis.

First, O-acyl urea contains a relatively basic nitrogen which might displace the acyl group according to:

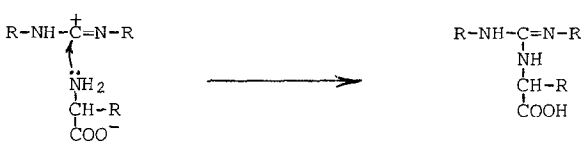


O-Acyl urea
(A transition state)

N-Acyl urea

N-Acyl urea is a recognized (1,6) by-product of carbodiimide-mediated peptide synthesis. It is expected only at low HCl concentration and should not appear when the concentration is high enough (in this case about 0.05 M) to protonate the urea moiety of O-acyl urea.

The other by-product probably originates from the carbonium ion form of protonated carbodiimide (Figure 1). The latter can be attacked by the amino instead of the carboxylate group. Such a reaction would lead to a guanido derivative.



The observed formation of a second product in 0-0.01 M HCl also containing carbodiimide and amino acid but unaccompanied by dipeptide indicates that this

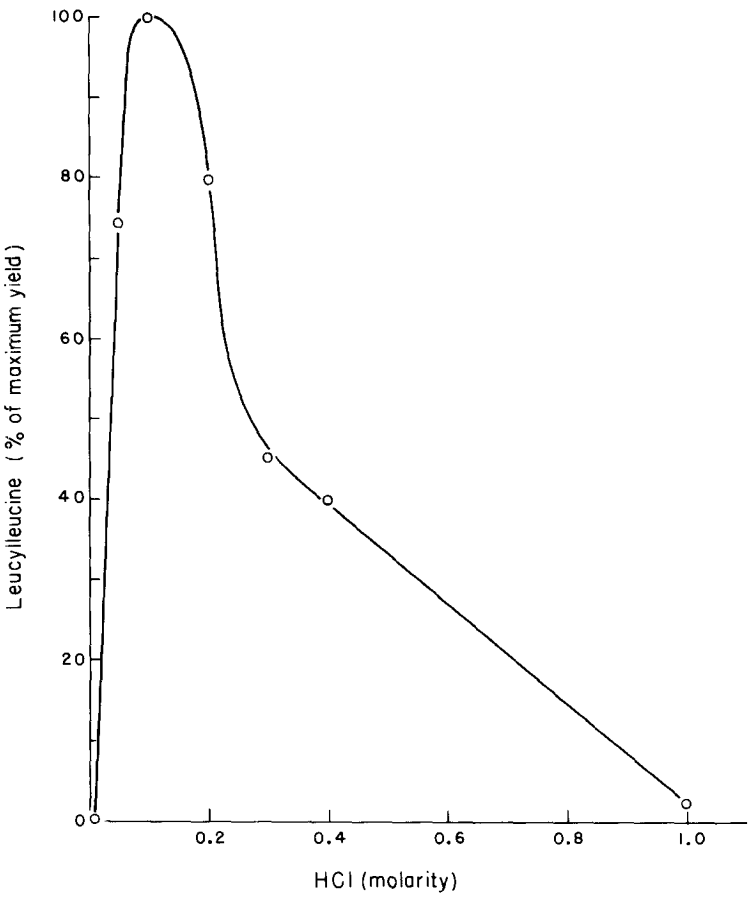



Fig. 2. Leucylleucine production as a function of acid concentration.

acid concentration controls the reaction prior to formation of O-acyl urea. This effect may account for guanido derivatives.

Spontaneous hydrolysis of O-acyl urea to a urea and free amino acid offers yet another possible side-reaction. However, extensive peptide formation at this relatively low amino acid concentration and moderate HCl concentrations suggests that this process is probably minor.

Acid concentration in Figure 2 varied on both sides of that of condensing agent. According to the mechanism of Figure 1, two equivalents of protons are required in the first stages of peptide synthesis. Therefore, at acid concentration much greater than that of carbodiimide, protonation of amino

acid should become essentially complete and block nucleophilic displacement from O-acyl urea by uncharged amino group.

Occurrence of maximum yield in 0.1 M HCl for 0.05 M carbodiimide is compatible with the first stages of the accepted pathway. However, it is disturbing that substantial amounts of dipeptide were obtained in 0.4 M HCl, an 8-fold excess. In this connection note that the accepted mechanism proposes release of the two equivalents of proton required in the early steps. This release prevents normal reaction from reducing the proton concentration. Furthermore, the transition state intermediates of Figure 1 have never been isolated; their structures have been inferred (2,3,6) from the known end product. Thus, it is possible that the original mechanism is only partly correct for aqueous systems. The change in slope of Figure 2 at high acid concentration suggests that another mechanism might participate under this condition. Perhaps a di-amino acid anhydride (3) contributes but is less available for peptide synthesis than the intermediates existing at lower HCl concentration.

Uncertainty about the mechanism makes desirable an investigation of carbodiimide condensations in aqueous systems. Meanwhile it is clear that acid concentration should be controlled during peptide synthesis.

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