Mechanism of Amide Formation by Carbodiimide for Bioconjugation in Aqueous Media

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To study the mechanism of amide formation between carboxylic acid and amine in aqueous media using 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDC), hydrogels with two different types of carboxyl group locations were employed as substrates containing the carboxylic acid, while ethylenediamine and benzylamine were used as amine. In parallel, a study was undertaken with cyclizable carboxylic acids (maleic acid and poly(acrylic acid) and noncyclizable carboxylic acids (fumaric acid and poly(ethylene glycol) with the terminal carboxyl groups) to assess the reaction products by $^{13}$C-NMR and IR. EDC rapidly lost its activity in aqueous media of low pH, producing the corresponding urea derivative, but was very stable at neutral and higher pH regions. EDC could react with carboxyl groups at a relatively narrow low pH range such as 3.5-4.5. If carboxyl groups were cyclizable, they would react quickly with EDC producing carboxylic anhydrides, which formed the corresponding amides when amine compounds were present. On the other hand, a trace of amide was formed in the case of noncyclizable carboxylic acids. In addition, an excess of EDC caused an undesired side reaction to form stable N-acylurea, regardless of the special location of carboxylic acids.

INTRODUCTION

Carbodiimides (RN=C=NR) are unsaturated compounds with an allene structure. Since their first synthesis from thioureas at the end of the last century, their physical and chemical properties are summarized in review articles (Kohrana, 1953; Kurzer & Douraghi-Zadeh, 1987; Williams & Ibrahim, 1981). Extensive studies have been also devoted to reactions of carbodiimides such as isomerization of themselves and addition reactions with water, alcohols, amines, phenols, and carboxylic acids. In addition, carbodiimides have been applied for peptide synthesis (Sheehan & Hlavka, 1956) and modification of polysaccharides (Danishefsky & Siskovic, 1971) and proteins (Wilchek et al., 1967; Hoare & Koshland, 1967) in aqueous systems.

Recently, attention has been paid to new synthesis and application of carbodiimides. For example, Gontar et al. synthesized perfluorodialkylcarbodiimides via the rearrangement of perfluorooazidomethines (Gontar et al., 1985). Synthesis of other carbodiimides was also reported (Gorbatenko et al., 1984; Matveev et al., 1988; Saito et al., 1992). Further, peptide synthesis (Kricheldorf et al., 1986; Kessler & Hutscher, 1986; Wang et al., 1987), inactivation of thrombin (Chan et al., 1988), Diels-Alder reaction (Trifonov & Orahovants, 1989), and modification of DNA (Dolinnaya et al., 1990, 1991) were investigated using carbodiimides.

Bioconjugation by carbodiimides has also been widely performed. Carbodiimides are very effective in modifying and crosslinking proteins such as lysozyme (Yamada et al. 1981), myosin (Onishi et al., 1989a,b), neurotoxin (Schmidt & Betz, 1989), actin (Takashi, 1988), F$_1$ adenosine triphosphatase (Bragg & Hou, 1986a,b), and cytochrome c and b$_6$ (Mauk & Mauk, 1989) because no residues remain in the crosslinked protein (zero-length crosslinker).

Most of the above-mentioned studies are based on amide formation under very mild conditions between carboxylic acids and amines in aqueous and organic systems in the presence of carbodiimides. However, surprisingly few studies have focused on the elucidation of mechanism of the amide formation, particularly in aqueous systems in spite of their great importance in bioconjugation. On the contrary, reactions of carbodiimides with carboxylic acids in organic systems have been intensively studied to find that carboxylic anhydrides and the corresponding urea derivatives are produced by the reaction, as cited in the references (Kohrana, 1953; Kurzer & Douraghi-Zadeh, 1987; Williams & Ibrahim, 1981). On the other hand, Swaisgood and Natake studied the reaction of carboxyl groups in 1-glutamate dehydrogenase with glycine methyl ester in an aqueous medium in the presence of carbodiimides and found that the pH of the reaction medium strongly affected the reaction under formation of undesirable side-products if a high concentration of carbodiimide was present (Swaisgood & Natake, 1973). Frequently, an excess of carbodiimide is used in the isolation of biological membranes (Jacobson, 1977; Kalish et al., 1978) and in modification of polysaccharides (Danishefsky & Siskovic, 1971) to ensure the reaction.

In 1967 Hoare and Koshland proposed a mechanism for the amide formation by carbodiimide in aqueous media, but their mechanism is inconsistent with that proposed in text books (Solomons, 1988; Wong, 1991). Carbodiimide is thought in these text books to react with the hydroxyl groups of carboxylic acid, whereas Hoare and Koshland stressed the reaction of carbodiimide with a proton.

In the present report we investigate the effects of various factors influencing the reaction with carbodiimide in an aqueous system, such as the effect of pH and the dissociation of carboxyl and amino groups, to get deeper insight into the role of carbodiimide in amide formation. For this reason we selected hydrogel having carboxyl groups as substrates to make the separation of the
**MATERIALS AND METHODS**

1. **Materials.** 1-Ethyl-3-(3-(dimethylamino)propyl)-carbodiimide (EDC, MW = 191.7), a water-soluble carbodiimide (WSC), was purchased from Dohjin Kagaku Co., Ltd., Kumamoto, Japan. It was a hydrochloride salt. Toluidine blue O (MW = 305.84) and acid orange 7 (MW = 350.33) were purchased from Chroma Gessellschaft Schmidt & Co., Germany and Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan, respectively. Poly(ethylene glycol)-dioglycolic acid (PEG—COOH, MW = 3000, free acid), poly(acrylic acid) (PAAc, MW = 4 × 10^6, sodium salt), maleic acid (free acid), fumaric acid (free acid), ethylenediamine, ethanolamine, and deuterium oxide (D_2O) were obtained from Wako Pure Chem. Ind. Ltd., Osaka, Japan. Benzylamine and sodium 2,4,6-trinitrobenzenesulfonate (TNBS) were purchased from Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan. They were used without further purification. All the starting amine compounds were in free base form.

To study the effect of the location of carboxyl groups on amide formation, hydrogels were prepared by two different methods.

(A) Noncyclizable: 100 mL of aqueous solution containing 20 g of acrylamide, 100 mg of N,N'-methylenebis-(acylamide), and 200 mg of ammonium peroxodisulfate as the polymerization initiator was kept at 0 °C, and then 0.05 mL of the solution was poured into a dish of 6.4 mm diameter (Corning) after an addition of 0.2 mL of 4% acrylamide aqueous solution containing the same reagents as in A, and the pH was adjusted to 7.0. Polymerization was allowed to proceed in the same manner as the A, but without hydrolysis.

The concentration of carboxyl groups in the gels was estimated by staining with 5 × 10^−4 M toluidine blue at 25 °C and pH 10.0 for 3 h for both the gels. After being rinsed three times with water at pH 10.0, the bound toluidine blue was extracted with 50 v/v % acetic acid. The absorbance of the dye was measured at 633 nm to determine the concentration of carboxyl groups under the assumption that the number of bound dye molecules is equal to the number of carboxyl groups in gels according to the method reported elsewhere (Uchida et al., 1993).

The hydrogels prepared by methods A and B were designated as Gel-A and Gel-B, respectively. The characteristics of these gels are summarized in Table 1.

2. **Determination of EDC.** The colorimetric assay reported by Jacobson and Fairman (Jacobson & Fairman, 1980) was modified to determine the carbodiimide concentration. Briefly, aqueous solution of EDC was mixed with pyridine buffer solution (2.0 M pyridine and 1.0 M ethylene diamine, pH 7.0) and the increase in the absorbance at 400 nm was measured as a function of time at 25 °C. The absorbance increased linearly with time, and the slope was determined as a function of EDC concentration. Apparently, the rate of absorbance enhancement increased quite linearly with the EDC concentration at least lower than 3.5 mg/mL (correlation coefficient = 0.9995).

3. **Reaction of Carboxylic Acids with EDC.** To investigate the reaction of carboxyl groups with EDC, Gel-A, PEG—COOH, and PAAc were chosen. The IR spectra of PEG—COOH and PAAc were recorded to assess the chemical structure.

**Table 1. Characteristics of Hydrogels Having Carboxyl Groups**

<table>
<thead>
<tr>
<th>gel sizea</th>
<th>amt of carboxylb</th>
<th>water contentc</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mm)</td>
<td>groups (nmol/piece)</td>
<td>(w/w %)</td>
</tr>
<tr>
<td>Gel-A</td>
<td>12.0 ± 2.2</td>
<td>1.347 ± 50</td>
</tr>
<tr>
<td>Gel-B</td>
<td>9.8 ± 1.7</td>
<td>138 ± 2</td>
</tr>
</tbody>
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a Diameter × thickness of swollen gel. b Evaluated by staining with toluidine blue and expressed as average ± S.E. (n = 6). c Expressed as average ± S.E. (n = 3). d Carbonyl groups were introduced into polyacrylamide gel by alkaline hydrolysis to 0.96 mol %. e Prepared by copolymerization of maleic acid and acrylamide.

products from the reaction batch easy. In addition, the difference in the location of carboxyl groups (cyclizable and noncyclizable) on amide formation was also investigated using hydrogels, water-soluble polymers, and low-molecular-weight molecules having carboxyl groups.

The colorimetric assay reported by Jacobson and Fairman (Jacobson & Fairman, 1980) was modified to determine the carbodiimide concentration. Briefly, aqueous solution of EDC was mixed with pyridine buffer solution (2.0 M pyridine and 1.0 M ethylene diamine, pH 7.0) and the increase in the absorbance at 400 nm was measured as a function of time at 25 °C. The absorbance increased linearly with time, and the slope was determined as a function of EDC concentration. Apparently, the rate of absorbance enhancement increased quite linearly with the EDC concentration at least lower than 3.5 mg/mL (correlation coefficient = 0.9995).

**Reaction of Gel-A.** Three pieces of water-swollen Gel-A were put into 20 mL of 10 mg/mL of EDC aqueous solution of different pHs and kept at 25 °C for 2 h. The concentration of carboxyl groups remaining after completion of the reaction was determined by a staining method with toluidine blue.

The stability of carboxyl groups in Gel-A treated with EDC was investigated as follows. After pretreatment of the gel with 100 mL of 10 mg/mL of EDC solution (in excess) at pH 4.5 and 25 °C for 2 h, the treated gels were subjected to the reaction with 30 mL of 5.0 v/v % acetic acid at different pHs and 25 °C for 16 h. The tert-amino groups in gels originating from EDC residues were determined by staining with acid orange 7 as reported elsewhere (Uchida et al., 1993). Briefly, the specimen was stained with 5 × 10^−4 M acid orange 7 at pH 3.0 and 25 °C for 3 h, and the bound acid orange was extracted with 30 v/v % ethanolamine after being rinsed three times with water at pH 3.0, and then the absorbance of the dye at 468 nm was measured to determine the concentration of the tert-amino groups.

**Reaction of PEG—COOH.** Two g of EDC (in excess) was added to 50 mL of 1.0 g/mL of PEG—COOH aqueous solution of pH 4.5 and the mixture kept at 25 °C for 60 min. The products were recovered by dialysis of the solution against water for 48 h at 25 °C, followed by freeze-drying.

**Reaction of PAAc.** A 0.5 g portion of EDC was added to 50 mL of 1.0 g/mL of aqueous PAAc solution of pH 4.5 and reacted at 25 °C for 5 min ([EDC] = [−COOH]/2). The products were recovered from the solution by reprecipitation with 500 mL of dried acetone and vacuum-drying at 25 °C.

The IR spectra of PEG—COOH and PAAc treated with EDC were recorded by FTIR-8100, Shimadzu Inc., Kyoto, Japan.

**4. Amide Formation. Reaction of Hydrogels.** Amide formation between a carboxyl and an amino group in the presence of EDC was investigated using Gel-A and Gel-B with two different methods. The one included two steps. Thirty pieces of gel were put into 100 mL of 0.1 M acetic acid at pH 4.5. Following an addition of 3.0 mL of 0.33 g/mL of EDC aqueous solution to the acetic acid solution ([EDC] = [−COOH]/2), the reaction was allowed to proceed for a given period of time at 0 °C. Then, these gels were taken out from the solution and immediately placed in 30 mL of aqueous amine solution (0.1 M ethylenediamine or benzylamine in 0.1 M NaH_2PO_4) at various pHs and further reacted for 30 min at 25 °C. Three pieces of gels were used for the reaction batch of each pH.
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The other method was one step. Gel was put into 30 mL of a mixture of 0.1 M ethylenediamine, 0.1 M NaH2PO4, and 0.01 M acetic acid at different pHs. After an addition of 0.72 mL of 40 mg/mL of aqueous EDC solution, the solution was kept at 25 °C for 30 min ([EDC] = [-COOH]/2). Three pieces of gels were used for the reaction batch of each pH.

The extent of amide formation was determined by measuring the decrease in carboxyl group in the gels with the staining method using toluidine blue. For each of the reactions with gels, the initial concentration of EDC was kept to one half of the molar concentration of total carboxyl groups involving acetic acid used to maintain the pH of solution constant.

Reaction with Small Carboxylic Molecules. Amide formation from maleic and fumaric acids was investigated as follows. A 0.5 g portion of maleic or fumaric acid and 1.0 g of ethylenediamine were dissolved in 50 mL of water. After an addition of 0.826 g of EDC ([maleic acid] = [fumaric acid] = [EDC]), the pH was adjusted to 5.0 and the reaction was allowed to proceed at 25 °C for 1 h. The reaction was stopped by an addition of 1.0 mL of 1 N HCl. A similar reaction was performed using the deactivated EDC (0.826 g of EDC was dissolved in 3.0 mL of 1 N HCl and hydrolyzed for 3 h at 25 °C). The products were recovered by freeze-drying, and 13C-NMR spectra were recorded in D2O (JEOL Co. Ltd., GSX-270, Tokyo, Japan).

The degree of amide formation was estimated by amine determination. Briefly, 0.25 g of maleic or fumaric acid and 0.132 g of ethanolamine were dissolved in 40 mL of water, and the pH was adjusted to 7.0. After an addition of 5.161 mL of 80 mg/mL of aqueous EDC solution, the pH was adjusted to 7.0 again and the solution was diluted exactly to 50 mL. The reaction was carried out at 25 °C under the condition of [maleic acid] = [fumaric acid] = [EDC] = [ethanolamine]. One mL of the solution was taken out at a given time, followed by hydrolysis of EDC remaining by an addition of 1.0 mL of 1 N HCl. After neutralization with 1.0 mL of 1 N NaOH, the amount of amine residue was determined with the conventional TNBS method (Habeeb, 1966). Following 100 times dilution of the solution with water, 0.5 mL of solution, 10 mL of 1.0 mg/mL of aqueous TNBS solution, and 2.0 mL of 40 mg/mL of aqueous sodium bicarbonate (pH 9.0) were mixed and incubated at 37 °C for 2 h. After the mixture was cooled to 25 °C, the absorbance before and after amide formation was measured at 355 nm. In this assay, ethanolamine was used instead of ethylenediamine because TNBS formed a water-insoluble deposit with ethylenediamine.

RESULTS


The stability of EDC at different pHs and 25 °C is given in Figure 1 in terms of the carbodiimide activity remaining after incubation. Apparently, EDC is quite stable at the neutral and higher pH regions, at least, for 5 h at 25 °C. This is in good agreement with the result of protein crosslinking by EDC (data not shown). On the other hand, the activity of EDC decreased at low pHs. The product formed at lower pH regions was confirmed by IR as urea derived from the EDC (data not shown).

2. Reaction of EDC with Carboxyl Groups.

The pH dependence on the disappearance of carboxyl groups in Gel-A upon the reaction with EDC is given in Figure 2. The molar concentration of EDC was 260 times larger than that of carboxyl groups in order to modulate the pH of solution. The reaction markedly depended on pH, the optimal pH ranging between 3.5 and 4.5. The suppression of reaction at pHs lower than 3.5 should be ascribed to the decreased dissociation of carboxyl groups in Gel-A at low pHs. This suggests that proton and ionized carboxyl groups are required for the reaction with EDC. This reaction occurred only when an excess EDC was used, and no change in carboxyl groups was observed when the molar ratio of EDC to carboxyl groups was lower than 0.5.

The pH dependence of the stability of product yielded from the carboxyl groups in Gel-A and EDC is given in Figure 3. As can be seen, the reaction product was quite stable at pHs lower than 8, indicating that this product was too stable to form amide with amine.

IR spectra of PEG-COOH treated with EDC are given in Figure 4. The peak absorbance of the untreated PEG-COOH appearing at 1520 cm⁻¹ is assigned to the C=O stretching vibration of COO⁻. Clearly, this peak became strikingly lower upon reaction with EDC and new peaks appeared at 1630 cm⁻¹ and 1530 cm⁻¹ assigned to C=O stretching vibration and NH bending vibration of amide, respectively. These results suggest that a
Figure 3. pH dependence of stability of the product formed between carboxyl groups in Gel-A and EDC. Thirty pieces of Gel-A were placed in 100 mL of 10 mg/mL of EDC aqueous solution of pH 4.5 at 25 °C for 2 h. Then, three pieces were taken out and treated with 30 mL of 5 v/v% acetic acid aqueous solution of various pHs at 25 °C for 16 h. Data are the average of three readings.

Figure 4. IR spectra of PEG-COOH treated with EDC (A) and untreated PEG-COOH (B). Both the samples were cast from the chloroform solution on a KBr plate.

stable amide such as N-acylurea is formed as a result of the reaction of carboxyl groups in PEG-COOH with carbodiimide. In this reaction the molar concentration of EDC was about 30 times larger than that of carboxyl groups in PEG-COOH. This stable amide was observed only when such an excess of carbodiimide was used.

IR spectra of the reaction products of PAAc with EDC are given in Figure 5. New peaks appeared at 1750, 1800, and 1050 cm⁻¹ when PAAc reacted with EDC. They may be assigned to C=O and C–O–C stretching vibrations. The spectrum changed to that of the virgin PAAc when PAAc was again dissolved in water after reaction with carbodiimide (B). It is likely that the reaction of carboxyl groups in PAAc with EDC yielded carboxylic anhydride, which was highly hydrolyzable by water. In this reaction the molar concentration of EDC was about two times less than that of carboxyl groups in PAAc.

4. Amide Formation. Reaction of Hydrogels. Amide formation between carboxyl groups in gels and ethylenediamine was investigated using “one-step” and “two-step” methods. The results are given in Table 2. Clearly, carboxyl groups in Gel-B were more effective in formation of amide than those in Gel-A for both the methods. In addition, the extent of amide formation with the one-step method was higher than that with the two-step method, regardless of the gel type. However, it should be noted that the extent of amide formation with the one-step method was much larger than 50% in the case of Gel-B, although the molar ratio of EDC to the carboxyl group was 0.5. Therefore, the staining method by toluidine blue could only semiquantitatively evaluate the degree of amide formation.

The pH dependence of the amide formation was investigated using Gel-B with the two different methods. The results are given in Figures 6 and 7 for the two-step and the one-step method, respectively. As can be seen in Figure 6, amide formation was promoted with the increasing pH for the “two step” method, when benzylamine was employed as amine. On the other hand, such a prominent pH effect was not observed in the case of ethylenediamine, although the reaction extent was much higher than that with benzylamine throughout the pH range studied.

In the case of the one-step method the optimal pH for amide formation was observed around 5 as shown in Figure 7. This is different from the result of the “two step” method. As mentioned above, carbodiimide can react with carboxyl groups in gel only at a low pH region from 3.5 to 4.5. As amine molecules are required for amide formation to be in an unionized state, it is likely
Figure 6. Amide formation from Gel-B and two different amines with the two-step method. Thirty pieces of Gel-B were placed in 100 mL of a mixture of 0.1 M acetic acid and 1.0 g of EDC at pH 4.5 and 0 °C for 30 min. Then, the gels were put in 0.1 M ethylenediamine or 0.1 M benzylamine in 0.1 M NaH2PO4 at various pHs and 25 °C and allowed to react for 30 min. Data are the average of three readings: (○) ethylenediamine, (●) benzylamine.

Figure 7. Amide formation from Gel-B and ethylenediamine with the one-step method. Three pieces of Gel-B were placed in 30 mL of mixture of 0.1 M ethylenediamine, 0.1 M NaH2PO4, and 0.01 M acetic acid at various pHs and allowed to react at 25 °C for 30 min after an addition of 0.72 mL of 40 mg/mL of EDC aqueous solution. Data are the average of three readings.

Figure 8. Pretreatment time dependence of the amide formation from Gel-B and ethylenediamine with the two-step method. Thirty pieces of Gel-B were placed in 100 mL of a mixture of 0.1 M acetic acid and 1.0 g of EDC at 0 °C and pH 4.5 for different periods of time. Then, three pieces were taken out and immediately put in 0.1 M ethylenediamine in 0.1 M NaH2PO4, followed by reaction at pH 7.0 and 25 °C for 30 min. Data are the average of three readings.

Figure 9. 13C-NMR of maleic acid (A) and fumaric acid (B) treated with ethylenediamine in the presence of EDC at 25 °C for 1 h. A 0.5 portion g of maleic or fumaric acid and 1.0 g of ethylenediamine were dissolved in 50 mL of distilled water. After an addition of 0.826 g of EDC, the pH was adjusted to 5.0 and the reaction was allowed to proceed at 25 °C for 1 h. The reaction was stopped by an addition of 1.0 mL of 1 N HCl. Products were recovered by freeze-drying and dissolved in D2O for 13C-NMR measurement.

In the case of fumaric acid, the product with the highest content was the nonreacted acid. On the other hand, the content of the nonreacted acid was relatively low in maleic acid. It is likely that maleic acid yielded a larger amount of amide than fumaric acid, though three other peaks could not be assigned. The peaks of carbonyl carbons at around 170 ppm also could not be assigned (data not shown).

Figure 10 shows the extent of amide formation between maleic or fumaric acid and ethanolamine as a function of reaction time. Maleic acid could react rapidly with
ethanolamine and about 80% of amide formation was observed within 1 h, whereas the reaction of fumaric acid was very low and the yield was as low as about 30% even after 24 h. Such a considerable difference in amide formation between maleic and fumaric acids might be ascribed to the conformation of carboxyl groups in the molecules.

DISCUSSION

As shown in Figure 1, carbodiimide was hydrolyzed at low pHs such as 2.52 and 3.95, suggesting that proton is required for hydrolysis in aqueous media, though Knore et al. reported that hydrolysis of carbodiimide was catalyzed also by hydroxide ions as cited in the reference (Williams & Ibrahim, 1981). It is likely that the reaction rate constant of hydrolysis in the acidic media is much higher than in the basic media. The reaction of carbodiimide with carboxyl groups proceeds most rapidly in the pH range from 3.5 to 4.5, as shown in Figure 2. This indicates that both of protons and dissociated carboxyl groups are necessary for the reaction of carbodiimide.

As protein (Swaisgood & Natake, 1973) and polysaccharides (Danishefsky and Siskovic, 1971) possess a variety of functional groups such as \(-\text{NH}_2, -\text{OH}, -\text{SH}, \text{ and } -\text{COOH},\) which will make the passway of reaction with carbodiimide very complicated, we used hydrogels having carboxyl groups alone. One can separate the reaction products any time just by removing the gels from the reaction batch. The reaction product of the excess carbodiimide with carboxyl groups in gel and PEG-\text{COOH} may be \(N\)-acylisourea, which is markedly stable in aqueous media, as shown in Figure 3, and cannot produce amide even when amine is added to this compound. Swaisgood and Natake also reported that \(N\)-acylisourea was formed only when an excess of carbodiimide was used in comparison with carboxyl groups (Swaisgood & Natake, 1973). Indeed, no change in carboxyl concentration was observed for PEG-\text{COOH}, PAAc, Gel-A, and Gel-B in the absence of amine when the concentration of carbodiimide was two times lower than that of carboxyl groups.

The extent of amide formation in Gel-B was much larger than that in Gel-A as shown in Table 2. The different reaction extent may be explained in terms of location of carboxyl groups along the polymer chains in the gel. As the carboxyl groups in Gel-B were originated from maleic acid introduced into the gel by its copolymerization with acrylamide, it probably is easy for Gel-B to form carboxylic anhydrides. On the other hand, the carboxyl groups in Gel-A were introduced by random hydrolysis with NaOH, resulting in random location of the carboxyl groups along the polymer chains because the extent of hydrolysis was as low as 0.96 mol % as shown in Table 1. Thus, it is unlikely to form carboxylic anhydrides between two neighboring carboxyl groups in Gel-A, where the carboxyl groups are present more separately from each other than in Gel-B. This result well agrees with that of the reaction of noncyclizable PEG-\text{COOH} and cyclizable PAAc.

No anhydride was formed between carboxyl groups in Gel-A and acetic acid used as a buffer molecule. This is probably because no amide formation was virtually observed for this gel when the two-step method was employed, as shown in Table 2, and generally carboxylic anhydride forms amide by an addition of amine. It follows that formation of carboxylic anhydride is essential as an intermediate for production of the corresponding amide by an addition of amine for the two-step method.

On the contrary, the one-step method yielded a small extent of amide even if Gel-A was used. It is probable that a very small fraction of carboxyl groups in Gel-A could participate in direct amide formation with amine without passing through anhydride formation because anhydride was too difficult to form in Gel-A. This may be a reason for the larger extent of amide formation for the one-step method than for the two-step method, where the intermediate carboxylic derivative is required to remain in an active state until the counterpart amine comes. This result agrees with that of the reaction of small molecules (maleic and fumaric acids) with amine in the presence of EDC. Cyclizable acid (maleic acid) formed a much larger amount of amide than noncyclizable acids (fumaric acid) as shown in Figure 10.

The above findings allow us to propose the following reaction mechanism for the amide formation in aqueous media with the use of carbodiimide in terms of carboxyl group location, although exact structural information could not be obtained because of difficulty in isolation of products. As illustrated in Figure 11, a carbodiimide molecule reacts with a proton to form a carbocation. This reaction mechanism well agrees with that proposed by Hoare and Koshland (Hoare & Koshland, 1967) which is based on the reaction in an organic system reported by Khorana (Khorana, 1953) though inconsistent with that by others (Solomons, 1988; Wong, 1991).

However, the reaction mechanism of carbodiimide with carboxylic acid is more complicated in aqueous media than in organic systems. Unless carboxylic acid is present, 1 will be hydrolyzed by water into the corresponding urea derivative 2. 1 can react with an ionized carboxyl group to form a compound 3 (O-acylisourea). As a result of reprotonation at the site of Schiff base, 3 will change into a carbocation 4, followed by the attack of various bases present in the aqueous system. If any nucleophile is absent, 4 will transfer into the corresponding urea derivative 2 by the reaction with water. Since an ionized carboxyl group is a very strong base, its reaction with 4 may produce carboxylic anhydride 8 in the case of cyclizable carboxyl group, which quickly forms the corresponding amide 7 when amine is present. On the other hand, in the case of noncyclizable carboxyl group, 4 will react with a water molecule or unionized amine to yield carboxylate 6 or amide 7, respectively. The reaction with water must be prevailing, because the water concentration is much higher than that of amine and most of the amine molecules are in the ionized form.

**Figure 10.** Amide formation between maleic or fumaric acid and ethanolamine in the presence of EDC. A 0.25 g portion of maleic or fumaric acid and 0.132 g of ethanolamine were dissolved in 40 mL of water and pH was adjusted to 7.0. After an addition of 5.161 mL of 80 mg/mL of EDC aqueous solution, the pH was adjusted to 7.0 again and the solution was exactly diluted to 50 mL. The reaction was allowed to proceed at 25 °C. The extent of amide formation was determined by the TNBS assay: (○) maleic acid, (●) fumaric acid.
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in the presence of carbodiimide. Figure 11. Proposed reaction mechanisms of the amide formation between carboxylic acid and amine in aqueous media in the presence of carbodiimide.

at the low pH region which is required for the reaction of carbodiimide with carboxyl groups. Therefore, quite a few amine molecules can react directly with 4 to form 7 without anhydride formation. In this connection, the reaction scheme of carbodiimide in aqueous systems is quite different from that in organic systems. The pathway (4 → 8 → 7) was not mentioned by Hoare and Koshland. In addition, carbodiimide would be able to react with compound 4 to form N-acylurea 5 as byproduct, if carbodiimide is present in excess. As amide was formed more readily at higher pHs, and as shown in Figure 6, it is likely that only unionized amine molecules can react with the carboxyl groups pretreated with carbodiimide. The difference in the extent of amide formation between ethylenediamine and benzylamine in Figure 6 might be ascribed to the difference in the dissociation constant. As ethylenediamine has pK_a of 6.85 and pK_b of 9.93, while benzylamine has pK_b of 9.60 at 25 °C, it is clear that the ionization of ethylenediamine is markedly suppressed even at low pHs compared with that of benzylamine.

In summary, it may be concluded that amide formation from carboxyl and amino groups in aqueous media in the presence of carbodiimide requires formation of carboxylic anhydride as intermediate. Noncyclizable carboxylic acids cannot form a larger amount of amide than cyclizable carboxylic acids. The anhydride will be formed from carboxyl groups if carbodiimide is present at less than half of the molar concentration of carboxyl groups. The pH of solution strongly affects the reaction of carbodiimide with carboxyl groups. A narrow pH range such as 3.5–4.5 is the most favorable for the formation of carboxylic anhydride, while higher pH is preferred for amide formation to suppress the ionization of amine.

Finally, it should be noted that the two-step method, in which carboxylic anhydride is first prepared from two carboxyl groups by carbodiimide, followed by reaction with amine, may give a variety of selective reactions such as bioconjugation of enzymes to substrates having carboxyl groups without significant deactivation of enzymes, although the coupling efficiency is lower than that by the one-step method.

LITERATURE CITED


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