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Co-operative Effects of Functional Groups in Peptides. I. Aspartyl-serine Derivatives

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The β-benzyl esters of N-carbobenzyloxyaspartyl amides and peptides were found to undergo rapid saponification in dioxane–water mixtures. A study of the saponification rates of a number of these derivatives revealed a strong rate dependence on the nature of the particular amide or peptide. A general mechanism for these saponifications involving the formation of an imide intermediate is proposed. The intermediate has been detected both by direct isolation and by the changes in optical rotation accompanying the reaction. The most rapid rates of reaction measured, in respect to both the formation of the imide intermediate and its subsequent hydrolysis, were obtained with β-benzyl-N-carbobenzyloxyaspartylseryl amide. In 7:3 (v./v.) dioxane–water, the second-order specific rate of hydroxyl-ion catalyzed formation of benzyl alcohol from this compound exceeds, *by a factor of about* 10^7, the second-order specific rate of saponification of benzyl propionate. Although this rapid rate is dependent on the acidity of the peptide bond, the conformation of the molecule and the acidity of the seryl hydroxyl play important roles in the catalysis. The dependence of the reaction rates on the dielectric constant of the medium suggests a mechanism of imide formation and hydrolysis different from the mechanism of alkaline solvolysis of other carboxylic derivatives (esters, lactones, amides, analides and peptides), and involving tetrahedral intermediates. The significance of both the formation and cleavage of aspartyl imides to protein and polypeptide chemistry, and the special reactivity of aspartyl-seryl derivatives to the chemistry of the active sites of enzymes, are discussed.

Introduction

Studies on the effect of molecular structure on the rate of homogeneous solution reactions have dealt *primarily* with reactions involving the interaction of monofunctional catalysts and substrates. It has become increasingly suggestive that in biological reactions more than one functional group within the enzyme molecule participates in the catalytic interaction with the substrate.[5–7] The functional groups in the side chains of proteins which might possibly be associated with catalytic activity in aqueous media[8,9] are carboxylate ions (aspartate and glutamate), amines (lysine), guanido groups (arginine), phenolic groups (tyrosine), alcoholic hydroxyls (serine, threonine), thiols (cysteine) and the imidazole ring (histidine). In addition, the peptide bonds of the main chain may also participate in the chemical catalysis.

The investigation of catalysis involving two or more functional groups in the catalyst molecule has been reported in only a limited number of cases. Swain and Brown[10] found that 2-hydroxypyridine was far more effective than pyridine plus phenol as catalyst in the concerted general acid–general base catalyzed mutarotation on tetramethylglucose in aprotic solvents. Bender and co-workers[11,12] have reported that the monomethyl ester and monoamide of phthalic acid undergo hydrolysis *via* intramolecular catalysis at rates which are far more rapid than those observed for ester and amide hydrolysis with intermolecular catalysis. Bruice and Sturtevant[13,14] found that the *p*-nitrophenyl ester of γ-(4-imidazolyl)-butyric acid undergoes rapid hydrolysis as compared with the rate of imidazole-catalyzed hydrolysis of *p*-nitrophenyl acetate. Morawetz and collaborators[15–17] studied the hydrolysis of phenyl esters and anilides involving intramolecular catalysis by carboxylic acid or carboxylate in both low and high molecular weight materials. In all of the above examples of intramolecular catalysis a 5- or 6-membered cyclic intermediate has been either found or assumed.

For a group of proteolytic and esteratic enzymes (such as trypsin, chymotrypsin and cholinesterase), multifunctional participation in the chemical catalysis has been clearly established. These enzymes have been shown to contain one unusually reactive serine residue. Thus for example, although there are 30 serine residues in the enzyme α-chymotrypsin, only one of these alcoholic hydroxyls reacts rapidly and specifically with diisopropyl phosphofluoridate to yield the enzymatically inactive diisopropyl-phosphochymotrypsin.[18–20] The role of the serine hydroxyl in catalysis is further emphasized by the finding that the reactive serine residue is acylated during the enzymatic hydrolysis.[21a,b] The *p*H–activity dependence of the above enzymes suggests, in addition, that a base with an apparent dissociation

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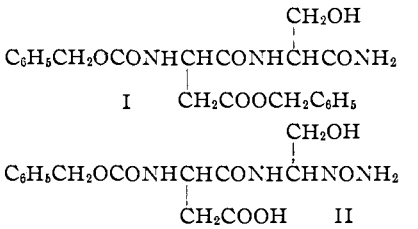
constant of about pK_a 7[22] is involved in the enzymatic catalysis. On the basis of the above facts, it has been postulated that the serine hydroxyl group and a basic group, such as the imidazole ring of histidine, participate in bifunctional enzymatic catalysis. A number of mechanisms of bifunctional catalysis involving these two groups either *via* electrophilic–nucleophilic,[5,6] or *via* general acid–general base catalysis[23,24] have been proposed. No model chemical reaction has been found, however, in which these two residues participate in multifunctional catalysis, although the reactions of hydroxylamine and tris-hydroxymethylaminomethane[25,26] with esters and the catalytic activity of 2-hydroxypyridine[10] with glycosides is suggestive of such possibilities.

Sequence analysis of peptides containing the active serine residue reveals the presence of a common sequence, *glycyl-aspartyl-seryl-glycine* in a number of these proteolytic and esteratic enzymes.[27] Of all the peptides for which the sequence analysis about the reactive serine has as yet been reported none has been shown to contain histidine. A 15-amino acid sequence in trypsin, in which the active serine is residue number 8, was shown to contain no histidine.[28] It thus can only be presumed that in the enzyme the active histidyl residue is located on a part of the peptide chain which is many chemical (peptide) bonds removed from the serine (but in stereochemical proximity), or else that histidine does not play an important role in the enzymatic catalysis and that the observed dependence of activity on *p*H is due to some other factor. Viswanatha and Liener[29] have reported that a fragment, isolated from a pepsin digest of acetyl-trypsinogen, having the catalytic activity of trypsin, contains no histidine.

Although a large peptide unit is probably necessary for enzymatic activity, we decided, on the basis of the unusual properties of the serine at the active site, to undertake a study of the chemical properties of some small peptides containing both serine and other potentially reactive amino acid residues. Both on the basis of the enzyme fragment studies mentioned above, and from the previous studies on multifunctional catalysis, the sequence -aspartyl-seryl- appeared to be of special enzymatic significance. Moreover, the studies of Edwards,[30] Garrett,[31] Brenner[32] and Rydon[33,34] on carboxyl derivatives (amide, peptide and oxazoline) indicated the possibility that the peptide bond itself might play an important role in multifunctional, intramolecular catalysis of acyl-transfer reactions. In view of these considerations, the synthesis of a peptide containing aspartic acid and serine (II) was attempted by coupling β-benzyl-N-carbobenzyloxy-L-aspartate with serine amide, then hydrolyzing the benzyl ester group of the resultant peptide derivative (I). The rate of benzyl ester hydrolysis was found to



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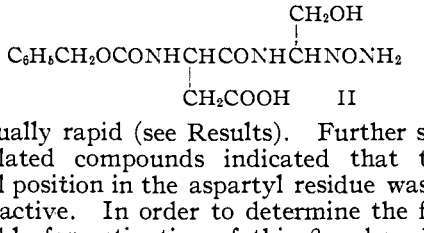

II

be unusually rapid (see Results). Further studies with related compounds indicated that the β-carbonyl position in the aspartyl residue was especially reactive. In order to determine the factors responsible for activation of this β-carbonyl position and to elucidate the mechanism of the hydrolytic reaction, a number or simpler β-benzyl-N-carbobenzyloxyaspartyl derivatives, lacking one or another of the functional groups of the seryl amide residue, were prepared and the hydrolysis of the β-benzyl esters were studied. From these studies it was concluded that in a peptide, the β-carbonyl of an aspartyl residue can acquire greatly enhanced reactivity due to coöperative, intramolecular catalysis involving peptide bonds and neighboring amino acid side chains (serine, in particular). Details are presented in the results and discussion below.

Experimental

All melting points reported are uncorrected.

β-Benzyl N-carbobenzyloxy-L-aspartate was synthesized according to Berger and Katchalski.[35]

β-Benzyl-N-carbobenzyloxy-L-aspartyl Amide (BENCAA)—To a solution of 3.57 g. (0.01 mole) of β-benzyl N-carbobenzyloxy-L-aspartate in 20 ml. of dimethylformamide at −5°, 0.01 mole of triethylamine was added followed by 0.01 mole of isobutyl chloroformate. The mixture was kept in the cold for several minutes and then poured into a solution of 0.67 ml. of concentrated aqueous ammonia (0.01 mole) in 20 ml. of dimethylformamide. The mixture was kept overnight at room temperature. On dilution with 500 ml. of cold water, a heavy precipitate formed. The precipitate was filtered and the filtrate was extracted with 50 ml. of ethyl acetate. The ethyl acetate extract was used to dissolve the precipitate. The ethyl acetate solution was in turn extracted twice with 1 *N* sodium bicarbonate and several times with water until no precipitate formed on acidification of the aqueous extract.

The ethyl acetate solution was dried over anhydrous sodium sulfate. White crystals formed when the solvent was evaporated *in vacuo*. The material was dissolved in ethyl acetate and recrystallized by addition of petroleum ether; yield 47%, m.p. 101°, $[\alpha]^{20}D$ +1.5° (*c* 2.5 in dioxane–water 1:1 v./v.). *Anal.* Calcd. for $C_{19}H_{20}N_2O_5$: C, 64.0; H, 5.7; N, 7.9. Found: C, 64.1; H, 5.8; N, 7.8.

β-Benzyl-N-carbobenzyloxy-L-aspartylmethyl amide (BENCAMA) was prepared by reacting the mixed anhydride of β-benzyl N-carbobenzyloxyaspartate and isobutyl chloroformate with 30% aqueous methylamine in dimethyl-

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formamide by the method described above for BENCAA; yield 50%, m.p. 116°, $[\alpha]^{20}D$ +2° (*c* 2 in dioxane–water 1:1 v./v.). *Anal.* Calcd. for $C_{20}H_{22}N_2O_5$: C, 64.9; H, 6.0; N, 7.6. Found: C, 65.0, H, 6.1; N, 7.6.

β-Benzyl-N-carbobenzyloxy-L-aspartylethanol amide (BENCAEA) was prepared similarly to BENCAA from the mixed anhydride of β-benzyl N-carbobenzyloxy-L-aspartate and isobutyl chloroformate using ethanolamine in dimethylformamide; yield 48%, m.p. 104°. *Anal.* Calcd. for $C_{21}H_{24}N_2O_6$: C, 63.0; H, 6.0; N, 7.1. Found: C, 63.0; H, 6.0; N, 6.9. When a dioxane solution of BENCAEA was titrated with perchloric acid in dioxane using thymol blue as indicator, no basic groups were found, thus demonstrating that the ethanolamine–aspartyl linkage is *via* an amide bond and not *via* an ester bond.

β-Benzyl-N-carbobenzyloxy-L-aspartylglycyl amide (BENCAGA) was prepared from the mixed anhydride of β-benzyl N-carbobenzyloxy-L-aspartate and isobutyl chloroformate and glycyl amide. Glycyl amide hydrochloride[36] and an equivalent amount of triethylamine were dissolved in 2 ml. of water and 20 ml. of dimethylformamide was added. The mixed anhydride was then added to this latter solution as in the preparation of BENCAA. BENCAGA was isolated and recrystallized by methods similar to those described for BENCAA; yield 45%, m.p. 126°. *Anal.* Calcd. for $C_{21}H_{23}N_3O_6$: C, 61.0; H, 5.6; N, 10.15. Found: C, 61.0; H, 5.5; N, 10.0.

L-Seryl Amide Hydrochloride.—L-Serine methyl ester hydrochloride (3.5 g.; prepared by the Fischer method[37]) was dissolved in 100 ml. of liquid ammonia in a wide-mouthed sealed tube. After 2 days, the excess ammonia was evaporated at room temperature and the residue dissolved in water. Residual ammonia, as well as the water, was removed by evaporation in a desiccator over sulfuric acid. This treatment was repeated three times. The material was then dissolved in methanol and precipitated with ether. White crystals were obtained; yield 57%. *Anal.* Calcd. for $C_3H_9N_2O_2Cl$: C, 25.6; N, 19.9; Cl, 25.2; neut. equiv., 140.5. Found: C, 24.9; N, 19.8; Cl, 25.0; neut. equiv., 139 as determined by anhydrous titration in ethanol with sodium methoxide using thymol blue as indicator.[38]

β-Benzyl-N-carbobenzyloxy-L-aspartyl-L-seryl amide (BENCASA) was prepared by the mixed anhydride method from β-benzyl N-carbobenzyloxyaspartate, isobutyl chloroformate and L-seryl amide hydrochloride similarly to BENCAGA; yield 35%, m.p. 157°, $[\alpha]^{20}D$ 1.7° (*c* 2.5 in dioxane:water 1:1 v./v.). *Anal.* Calcd. for $C_{22}H_{25}N_3O_7$: C, 59.6; H, 5.7; N, 9.5. Found: C, 59.0; H, 5.9; N, 9.1.

On anhydrous titration with perchloric acid no basic groups were found, demonstrating that serine is linked to aspartic acid by an amide bond and not by an ester bond. BENCASA was hydrolyzed with 6 *N* HCl at 110° for 12 hours. The hydrolyzate was analyzed chromatographically on paper for aspartic acid and serine. One μmole of peptide yielded 1 μmole of aspartic acid and 0.95 μmole of serine (corrected for the small loss of serine which occurs during acid hydrolysis).

β-Benzyl-N-carbobenzyloxy-D-aspartyl-L-seryl amide (D-BENCASA) was prepared by a procedure identical with that for L-BENCASA except that β-benzyl-N-carbobenzyloxy-D-aspartate was employed; m.p. 151°. *Anal.* Calcd. for $C_{22}H_{25}N_3O_7$: N, 9.5. Found: N, 9.3.

β-Benzyl-N-carbobenzyloxy-L-aspartyldimethyl amide (BENCADMA) was prepared similarly to BENCAMA, except that a concentrated aqueous solution of dimethylamine instead of methylamine in dimethylformamide was used. The final material could only be obtained as oil, $[\alpha]^{26}D$ −39° (*c* 2 in dioxane). *Anal.* Calcd. for $C_{21}H_{24}N_2O_5$: C, 65.6; H, 6.2; N, 7.3. Found: C, 64.5; H, 6.1; N, 6.4.

N-Carbobenzyloxy-β-alanine-benzyl ester was prepared according to Ben Ishai and Berger.[39]

β-Benzyl-N-carbobenzyloxy-L-aspartyl-L-serine methyl ester (BENCASME) was prepared from β-benzyl N-carbobenzyloxy-L-aspartate and L-serine methyl ester hydrochloride similarly to BENCASA; m.p. 121–123°. *Anal.* Calcd. for $C_{23}H_{26}N_2O_8$: C, 60.3; H, 5.7; N, 6.2. Found: C, 59.4; H, 5.8; N, 6.0.

α-N-Carbobenzyloxy-L-amino-(N-methyl)-succinimide.—BENCAMA (200 mg.) was dissolved in 5 ml. of dioxane and 5 ml. of Beckman standard borate buffer *p*H 10. The solution was acidified with 1 *M* hydrochloric acid until the *p*H meter read *p*H 9.3. The mixture was left at room temperature for 80 minutes and then diluted with 20 ml. of water. A small amount of precipitate formed which was removed by filtration. The aqueous solution was extracted with ethyl acetate, the extract dried over anhydrous sodium sulfate and the ethyl acetate removed by evaporation. The remaining residue was dissolved in 1 ml. of ethyl acetate and precipitated with petroleum ether. It was recrystallized from ethyl acetate–petroleum ether; m.p. 84°, $[\alpha]^{20}D$ −35° (1% in dioxane–water 1:1 v./v.). *Anal.* Calcd. for $C_{13}H_{14}N_2O_4$: C, 59.5; H, 5.34; N, 10.7. Found: C, 60.6; H, 5.54; N, 10.3.

Carbobenzyloxy-L-aminosuccinimide.—BENCAA (200 mg.) was dissolved in 10 ml. of 50% (v./v.) aqueous dioxane. The solution was brought to *p*H 11 on the *p*H-Stat (see below) and maintained at this *p*H until no further alkali was required. When this solution was neutralized with HCl and diluted with water, a precipitate formed. The precipitate was extracted with ethyl acetate and dried over sodium sulfate. The solvent was removed *in vacuo* and the residue crystallized twice from ethyl acetate–petroleum ether; m.p. 79°.

A sample was dissolved in ethanol and titrated with sodium methoxide using thymol blue as an indicator; neut. equiv. calcd. for $C_{12}H_{12}N_2O_4$ 248, found 251; $[\alpha]^{21}D$ −39° (*c* 1 in 95% ethanol). (The values cited in literature[40] are: $[\alpha]^{24}D$ −43° (*c* 1 in 95% ethanol) and m.p. 79–81°.)

N-Carbobenzyloxy-(α and β)-L-aspartyl-L-seryl Amides (CASA).—BENCASA (500 mg.) was dissolved in 2 ml. of methanol and 2 ml. of water was added. This solution was hydrolyzed in the *p*H-Stat at *p*H 10. One equivalent of NaOH was taken up within a few minutes. The mixture was acidified with HCl to *p*H 2, and water was added. The precipitate formed was filtered and recrystallized from methanol–water; m.p. 190°. *Anal.* Calcd. for $C_{15}H_{19}N_3O_7$: N, 119. Found: N, 12.0. This material constitutes a mixture of α- and β-peptides (see Results).

N-Carbobenzyloxy-(α and β)-L-aspartylglycyl Amides (CAGA).—This mixture was prepared from BENCAGA in a manner similar to that used in the preparation of CASA from BENCASA, except that the hydrolysis of the benzyl ester was carried out in the *p*H-Stat at *p*H 12; m.p. 185°. *Anal.* Calcd. for $C_{14}H_{17}N_3O_6$: C, 52.0; H, 5.3; N, 13.0. Found: C, 52.0; H, 5.3; N, 11.7. The material is a mixture of α- and β-peptides (see Results).

β-Ethyl N-Acetyl-L-aspartate.—The β-ethyl L-aspartate was prepared by the method of Piutti and Magli.[41] This compound (4.9 g.) was dissolved in 25 ml. of H_2O and cooled to 2°. The solution was brought to *p*H 9.0 with 4 *N* NaOH and maintained at this *p*H and temperature in a *p*H-Stat for 1 hour during which time 0.07 equiv. of acetic anhydride was added. The solution was then acidified to *p*H 2.5, evaporated *in vacuo* at 0° and the resultant precipitate extracted with 100 ml. of ethyl acetate. The product was precipitated on addition of petroleum ether and recrystallized from ethyl acetate–petroleum ether; m.p. 112–114°; equiv. wt. from potentiometric titration with base calcd. 203, found 198.

β-Ethyl-N-acetyl-L-aspartyl-L-seryl Amide (ETACASA).—The mixed anhydride from β-ethyl N-acetyl-L-aspartate and isobutyl chloroformate was prepared and coupled with L-serine amide under conditions similar to those employed in the synthesis of BENCASA. Addition of 0.1 *M* acetate buffer *p*H 5.0 at 0°, resulted in a clear solution (no precipitate). The mixed aqueous dimethylformamide solution was evaporated to dryness in a flash evaporator at 10°. The oily residue was extracted with 50 ml. of dimethylformamide and the product was precipitated by the addition of diethyl ether. The crude material was recrystallized from hot ethyl acetate; m.p. 124–126°.

Kinetic Method.—The following is a typical kinetic hydrolysis experiment: 0.01–0.05 mmole of reactant was dissolved in 1.67–2.50 ml. of dioxane in a reaction vessel.

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(40) E. Sondheimer and B. W. Holley, *J. Am. Chem. Soc.*, **76**, 2467 (1954).

(41) A. Piutti and G. Magli, *Gazz. chim. ital.*, **36**, 741 (1906).

Water was added to bring the final volume to 5.0 ml. A glass electrode and either an agar bridge connected to a calomel electrode or the electrode itself was immersed in the solution. The desired *p*H (in the range 8–12) was obtained by means of a *p*H-Stat (a combination of radiometer, type TTT 1a autotitrator and Ole Dich (Copenhagen) recorder). The addition of alkali (0.1–0.25 *N* NaOH), as a function of time, was recorded until the hydrolysis ceased. Blank rates, at the same *p*H and solvent medium, were determined and subtracted from the total rates. Blank rates were usually very small. In experiments requiring high precision (specific rates to ±1%), a water-jacketed (thermostated ±0.05°) reaction vessel was employed. Wherever the blank (CO_2 diffusion) rate was significant the vessel was swept with solvent-equilibrated nitrogen.

Potentiometric Titration of Hydrolyzates.—Immediately after a benzyl ester had been completely hydrolyzed, it was acidified with concentrated HCl to *p*H ~ 2.5, and a potentiometric titration was carried out by adding standardized NaOH. On calculating the *pK* values, the dioxane–water blank was subtracted from the alkali uptake. In both kinetic and potentiometric experiments, when precise control of the medium was required, 0.5 *M* NaCl was substituted for water to maintain quasi-constant ionic strength and the volume of base added was kept small relative to the reaction volume.

Optical Rotatory Changes during Hydrolysis.—The large changes observed during the course of hydrolysis of β-benzyl esters of aspartyl amides and peptides made this a particularly useful technique for the detection of intermediates during the reaction (see Results). A Rudolph model 90-S spectropolarimeter was used in most experiments. Since in these experiments there was no provision for "*p*H-stating," various buffers were employed, notably phosphate 0.1 *M*, *p*H 7.0; tris, 1.0 *M*, 0.5 *M* and 0.2 *M* *p*H 8–9; and borate buffers *p*H 8.8–10. The borate buffers were found to change (decrease in *p*H) during hydrolysis appreciably and hence could not be used for precision experiments. The concentration of reactants were chosen to optimize the optical rotation change without causing large changes in the *p*H of the solution. Path length varied from 1–4 decimeters. The solvents employed were the above-mentioned buffers mixed with dioxane (2:1 or 1:1 v./v. aqueous dioxane). For the most precise runs, both the polarimeter tube itself and the instrument trough into which it was placed were thermostated, by water-jacketing, to ±0.05°. Rotation readings were precise to from ±0.002° to ±0.01° depending both on the sample and the instrument.

Decarboxylation of the Hydrolysis Products of BENCAA and BENCAMA by N-Bromosuccinimide.—Amino acids are decarboxylated by N-bromosuccinimide.[42] On examination of the decarboxylation rate for asparagine and isoasparagine[43] it was found that the former releases CO_2 about seven times faster than the latter. This finding was used to estimate the relative amounts of α- and β-amide bonds in the hydrolysis products of BENCAA and BENCAMA (see Fig. 6).

The alkaline hydrolyzates of BENCAMA or BENCAA were acidified with hydrochloric acid and the carbobenzyloxy groups were removed by catalytic hydrogenolysis, using Pd–C as a catalyst.[43] After removal of the catalyst by filtration, the solution was decarboxylated by N-bromosuccinimide as reported previously.[42] The rate of decarboxylation was followed by measuring the amount of CO_2 evolved. Carbon dioxide-free nitrogen was bubbled through the reaction mixture in order to transfer the CO_2 formed into a 10% solution of benzylamine in ethanol–dioxane mixture.[44] The carbamic acid derivative which formed was titrated with sodium methoxide using thymol blue as indicator. From the rate of CO_2 evolution α- and β-peptides could be distinguished and their ratio determined (see Results).

Paper Electrophoresis of the Hydrolyzates of BENCAA and BENCAMA.—The BENCAA and BENCAMA hydrolysis products were decarbobenzyloxylated as described above. Drops of the solution as well as markers of asparagine, isoasparagine and aspartic acid were placed on Whatman No. 1 paper and electrophoresis was carried out in 0.05 *M* veronal buffer *p*H 8, at a potential gradient of 5 volt/cm.[45] After 4 hours the spots were detected by spraying with ninhydrin. Asparagine remained at the origin, isoasparagine moved 3.0 cm. toward the anode.

Analysis of the Optical Rotation–Time Curve.—Kinetic analysis of the optical rotation–time curves for the hydrolysis of L-BENCASA showed that the curves could be represented by the sum of two (first-order) exponential terms. If α is the optical rotation at any time, *t*, then

$$\alpha = Pe^{-k_1 t} + Q_e^{-k_2 t} + K$$

where *P* and *Q* are constants and k_1 and k_2 are the velocity constants shown in the scheme[46]

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

Although C is a mixture of two products (α- and β-peptides), a single constant k_2 can be used to describe the disappearance of B.

If the *molar rotations*, φ, of A, B and C are defined as $[\alpha_A]\cdot\bar{M}_A$, $[\alpha_B]\cdot\bar{M}_B$ and $[\alpha_C]\cdot\bar{M}_C$, respectively, where $\bar{M}$ = molecular weight × 10^{-3} (the values of $[\alpha_0]$ and ϕ_0 are the appropriate averages), the total rotation, α, at 10-cm. path length is given by

$$\alpha = [A]\phi_A + [B]\phi_B + [C]\phi_C$$

hence

$$\alpha = \frac{A_0}{k_2 - k_1}\{(\phi_A - \phi_C)k_2 + (\phi_B - \phi_A)k_1\}e^{-k_1 t} + \frac{A_0}{k_2 - k_1}(\phi_0 - \phi_B)k_1 e^{-k_2 t} + A_0\phi_C \quad (1)$$

where A_0 is the initial molar concentration of A, and

$$A_0\phi_C = K = \alpha \text{ at infinite time}$$

At low *p*H (0.2 *M* acetate, *p*H 5.0), ϕ_A could be readily determined since both k_1 and k_2 are very small; k_2 was determined on the assumption that at late times during the kinetic runs at higher *p*H, the contribution of the first term in eq. 1 is negligible. This assumption is based on the fact that in cases where the intermediate was isolated (see above), its specific optical rotation was approximately that calculated from the maximum value of α observed during the kinetic run ($\alpha_{max} \geq 0.7\ \alpha_{intermediate}$), hence $k_1 > k_2$. At these late times, the reaction was first order and the rate constant, k_2, could be calculated.

Equation 1 now contains two unknown parameters, ϕ_B and k_1. At the maximum in the α *vs.* *t* curve, $d\alpha/dt = 0$; hence

$$\phi_B = \frac{\{\phi_A(k_1 - k_2) + \phi_C k_2\}e^{-k_1 t_{max}} - \phi_C k_2 e^{-k_2 t_{max}}}{k_1 e^{-k_1 t_{max}} - k_2 e^{-k_2 t_{max}}} \quad (2)$$

and substitution of eq. 2 into eq. 1 yields

$$\phi_B = \left\{\frac{\alpha_{max}}{A_0} - \phi_C(1 - e^{-k_2 t_{max}})\right\}e^{-k_2 t_{max}}$$

The first specific rate, k_1, can now be calculated from eq. 2, *viz.*

$$\{k_1(\phi_B - \phi_A) - k_2(\phi_C - \phi_A)\}e^{-k_1 t_{max}} = k_2(\phi_B - \phi_C)e^{-k_2 t_{max}}$$

In these cases, $(\phi_B\text{-}\phi_A) > (\phi_C\text{-}\phi_A)$ and $k_1 > k_2$; hence to a good approximation

$$k_1 e^{-k_1 t_{max}} \simeq k_2\{(\phi_B - \phi_C)/(\phi_B - \phi_A)\}e^{-k_2 t_{max}}$$

The value of k_1 can then be refined by a second approximation. A theoretical curve of α *vs.* *t*, during the hydrolysis of L-BENCASA with $k_1 = 7.72 \times 10^{-2}$ min.$^{-1}$ and $k_2 = 6.64 \times 10^{-3}$ min.$^{-1}$ is compared with the experimental data in Fig. 1.

Kinetic Analysis of the "*p*H-Stat" Data.—If the hydrolysis of L-BENCASA is assumed to be the result of two consecutive first-order reactions, the concentration of the final product *C* at any time *t* is

$$C = \frac{A_0}{k_2 - k_1}\{k_2(1 - e^{-k_1 t}) - k_1(1 - e^{-k_2 t})\} \quad (3)$$

If only the reaction B→C (*i.e.*, the appearance of C) results

(42) E. W. Chappelle and J. M. Luck, *J. Biol. Chem.*, **229**, 171 (1957).

(43) M. Bergmann and L. Zervas, *Ber.*, **65B**, 1192 (1932).

(44) A. Patchornik and Y. Shalitin, *Bull. Res. Council Israel*, **5A**, 300 (1956), and *Anal. Chem.*, **33**, 1887 (1961).

(45) C. Ressler, *J. Am. Chem. Soc.*, **82**, 1641 (1960).

(46) T. M. Lowry and W. T. John, *J. Chem. Soc.*, **97**, 2634 (1910).

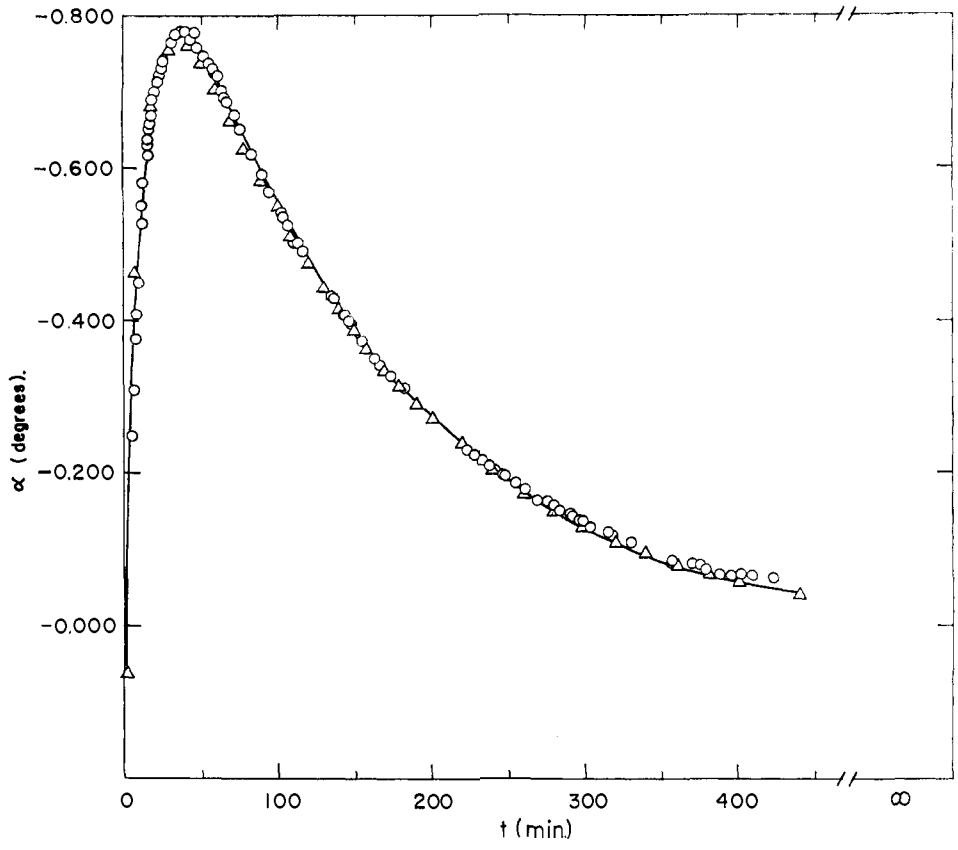


Fig. 1.—Optical rotatory changes (α) during the hydrolysis of L-BENCASA at *p*H 9.0; concentration was 10 mg./ml. in dioxane–0.4 *M* tris buffer (1:1 v./v.), in a 20-cm. path, 25°; the wave length of the polarized light was 436 mμ; O, experimental; Δ-calculated on the basis of two consecutive first-order reactions (see Experimental).

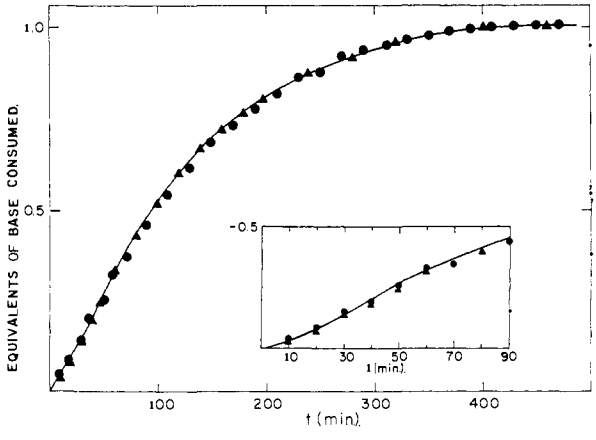


Fig. 2.—Alkali consumption during the hydrolysis of L-BENCASA (5 mg./ml.) in dioxane–0.4 *M* NaCl (1:1 v./v.) at *p*H 9.0, 25°. The insert is an enlargement of the early portion of the curve illustrating the "lag" in proton production: ●, experimental; ▲, calculated as the second of two consecutive first-order reactions.

in the liberation of protons, the rate of formation of C can be followed directly by the *p*H-Stat. At late times the reaction measured on the *p*H-Stat is first order; *i.e.*

$$\ln \frac{C_\infty}{C_\infty - C_t} = k_2 t$$

where C_∞ is the total amount of C formed at $t = \infty$, and C_t is that formed at time t. The values of k_2 obtained from

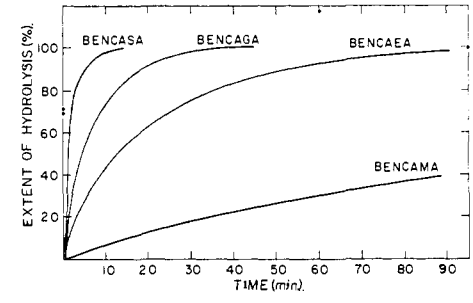


Fig. 3.—Alkali consumption during the hydrolysis of 0.01 *M* solutions of the β-benzyl esters of various N-carbobenzyloxy-L-aspartyl peptides in dioxane–water (1:2 v./v.), 24°, *p*H 10.5.

*p*H-Stat data agree well with those derived from optical rotation data. The course of hydrolysis of L-BENCASA at *p*H 9.0 in dioxane–water (1:1 v./v.) as measured in the *p*H-Stat is given in Fig. 2. The solid line represents the course of hydrolysis as calculated with the aid of eq. 3, assuming $k_1 = 0.062$ and $k_2 = 0.0087$ min.$^{-1}$ (see Table II).

Results

The time course of hydrolysis of the β-benzyl esters of some derivatives of N-carbobenzyloxy L-aspartic acid in 1:2 v./v. dioxane–water mixtures at *p*H 10.0 is shown in Fig. 3. The rates of hydrolysis of the β-benzyl esters of all the carbobenzyloxyaspartyl-*mono*substituted amides studied are unusually rapid relative to the rate of hydrolysis

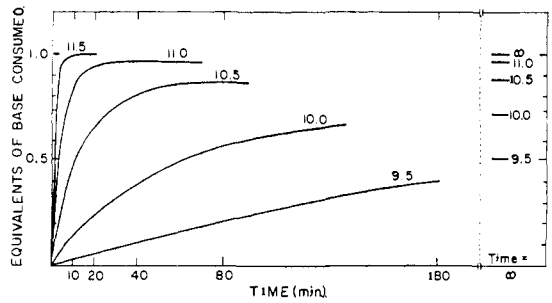


Fig. 4.—Alkali consumption during the initial reaction of BENCAA (see text) in dioxane–water (1:2 v./v.), 24°, at various *p*H's. Horizontal lines on the far right represent the equivalents of base consumed at (the extrapolated) infinite time.

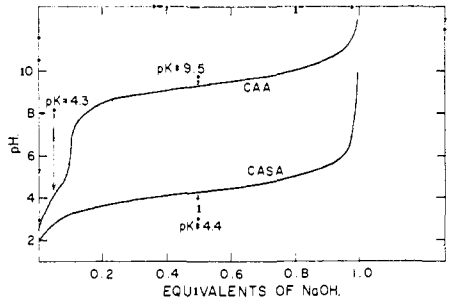


Fig. 5.—Titration curves of the reaction products of BENCAA (CAA) and BENCASA (CASA) in dioxane–water (1:2 v./v.). CAA and CASA were obtained after reaction for 1 hour at *p*H 11.0 and 10.0, respectively, at 24°.

of a disubstituted amide, BENCADMA, or a compound lacking the α-carboxamido group, N-carbobenzyloxy-β-alanine benzylester (see Table I).

TABLE I

HYDROLYSIS RATES[a] OF β-BENZYL N-CARBOBENZYLOXY-L-ASPARTATE DERIVATIVES IN DIOXANE–WATER (1:2 /v.v) AT 24° FROM *p*H-STAT DATA

Compound[b]	*p*H	$k_2 \times 10^2$, min.$^{-1a}$ 9.5	10.0	10.5	11.0
BENCAA					0.15[c]
BENCAMA			0.23	0.70	2.3
BENCAEA			1.15	3.5	20
BENCAGA		2.2	7	14	46
BENCASA		7	32	50	100
N-Cbz-β-alanine benzyl ester					0.05
BENCADMA					0.28
BENCAA (imide formation)		0.5	1.6	5.4	23

[a] Actually, the rate constants refer to the rate of hydrolysis of the corresponding imides in all but the last three cases (see Results and Discussion). [b] Initial substrate concentration was 0.01 *M*. [c] Obtained from optical rotation measurements on the assumption that the rate equation for hydrolysis of this compound is $v = k[\text{imide}^0][OH^-]$; the first-order rate constant must be corrected for the dissociation of the imide, *viz.*, $K = [\text{imide}^-][H^+]/[\text{imide}^0] = 3.2 \times 10^{-10}$ *M*. Hence for BENCAA, k_2 (first order) $= 4.9 \times 10^{-2}$ min.$^{-1}$.

β-Benzyl-N-carbobenzyloxy-L-aspartyl Amide (BENCAA).—The alkali uptake following the incubation of BENCAA in dioxane–water (1:2 v./v.) at various *p*H values is shown in Fig. 4. The total amount of alkali consumed varied with the *p*H at which the reaction was carried out,

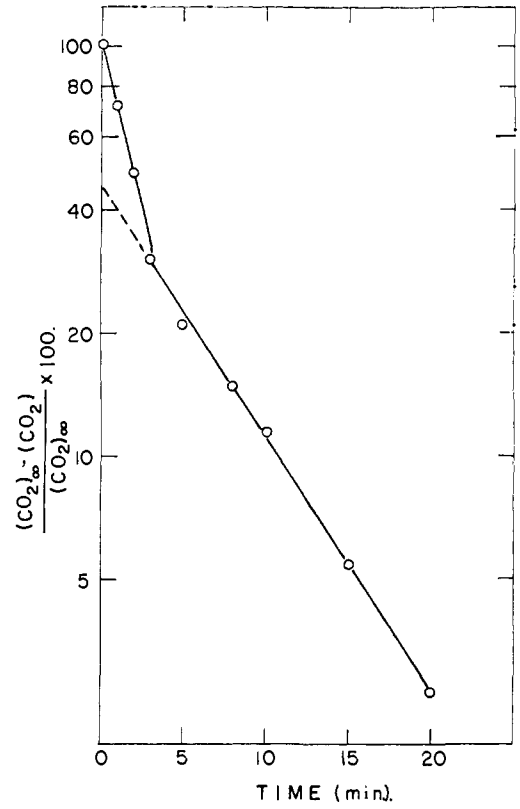


Fig. 6.—Decarboxylation of the decarbobenzyloxylated hydrolyzate of BENCAA by N-bromosuccinimide (see Experimental).

approaching a maximum of one equivalent at *p*H 11. At the end of the reaction (at *p*H 11), a potentiometric titration of the reaction products was carried out. A sample titration curve is shown in Fig. 5 (CAA). The titration curve obtained indicates that the principal product is a weak acid with pK_a 9.5 in dioxane–water (1:2 v./v.). A small amount of another product with pK_a 4.3 was also formed. The compound with pK_a 9.5 could be isolated from the reaction mixture on neutralization (see Experimental); it was identified as carbobenzyloxy-L-aminosuccinimide[40] and had a specific optical rotation $[\alpha]^{24}D$ −39° (*c* 1 in 95% ethanol). The large negative specific rotation of this cyclic compound distinguishes it from the linear compounds BENCAA, N-carbobenzyloxy-L-asparagine and -L-isoasparagine, all of which have small positive rotations ($[\alpha]^{24}D$ ~2–4°, in 95% ethanol). Although no chemical identification of the product with pK_a 4.3 was made, the results given below as well as those reported in the Experimental strongly suggest that it consists of a mixture of N-carbobenzyloxy-L-asparagine and N-carbobenzyloxy-L-isoasparagine, both derived from N-carbobenzyloxy-L-aminosuccinimide on hydrolysis. After two days incubation at *p*H 11 (dioxane–water 1:2 v./v.) a BENCAA solution was completely converted at room temperature to the acidic product with pK_a 4.3. An analysis of the reaction product thus obtained was carried out as follows: (a) The hydrolyzate was decarbobenzyloxylated by

catalytic hydrogenation and the product was treated with N-bromosuccinimide (see Experimental). From the rate of CO_2 evolution (see Fig. 6) it was concluded that approximately half of the product consists of a β-amide and the remaining half was an α-amide. (b) Paper electrophoresis of the same decarbobenzyloxylated BENCAA hydrolyzate in 0.05 M veronal buffer at pH 8 (see Experimental) revealed two spots of nearly equal intensity upon spraying with ninhydrin; one was identified as asparagine and the other as isoasparagine.

The kinetics of both the formation and the hydrolysis of the cyclic N-carbobenzyloxy-L-aminosuccinimide could be followed polarimetrically due to the above-mentioned differences in specific rotation of reactant, imide intermediate and products. In dioxane–water mixtures at pH $\geq$ 9.5, the rate of imide formation is much faster than the imide hydrolysis rate, and hence two nearly independent first-order optical rotation–time curves are obtained during the course of reaction, as illustrated in Fig. 7, curve A.

β-Benzyl-N-carbobenzyloxy-L-aspartyl-L-seryl Amide (BENCASA).—The time course of hydrolysis of BENCASA in dioxane–0.4 M aqueous NaCl (1:1 v./v.) at 25° and pH 9.5 is shown in Fig. 2. There is a short "lag" in the onset of proton production followed by a first-order rate of proton production over the rest of the time course. One equivalent of base is consumed by the end of the reaction. Following complete reaction the solution was acidified to pH 2.5 and a potentiometric titration of the reaction mixture was carried out with alkali. A sample titration curve is shown in Fig. 5 (CASA). The titration curve indicates that the principal product is an acid with pK_a 4.4. In order to elucidate the chemical structure of the alkaline hydrolyzate of BENCASA obtained at pH 9.5, the following experiment was carried out: Decarbobenzyloxylation was effected by catalytic hydrogenation and the products were chromatographed on Whatman No. 1 paper in a butanol–acetic acid–water mixture (4:1:4 v./v.). Two ninhydrin positive spots of almost equal intensity were obtained. The R_f of one of the spots was identical to that of an authentic sample of α-aspartylseryl amide (α-peptide, R_f = 0.19) obtained by the catalytic reduction of intact BENCASA; the other spot had an R_f of 0.13 and most probably corresponded to β-aspartylseryl amide (β-peptide). Paper electrophoresis, in 0.05 M veronal buffer, pH 8, of the catalytically reduced alkaline hydrolyzate of BENCASA resulted again in two ninhydrin positive spots as described in the Experimental. In connection with the above findings it is pertinent to note that decarbobenzyloxylation and debenzylation of BENCASA (by catalytic hydrogenolysis of BENCASA in dioxane) yielded a product which was chromatographically and electrophoretically pure by the criteria of the above-mentioned procedures.

The change in optical rotation with time for BENCASA in dioxane–0.4 M aqueous tris buffer (1:1 v./v.) solution at pH 9.00 and 25° is given in Fig. 1. This curve (Fig. 1) resembles that obtained with BENCAA (Fig. 7A). The presence of a maximum in the optical rotation–time curve is indicative of the formation of an intermediate with high levorotation. At least two consecutive steps are thus required to explain the hydrolytic reaction under discussion. The curve of Fig. 1 can be adequately represented by two rate parameters: one (k_1) for the formation of the intermediate compound, and one (k_2) for the degradation of this intermediate (see Experimental). The solid line in Fig. 1 is the expected change in optical rotation for $k_1 = 7.7 \times 10^{-2}$ min.$^{-1}$, $k_2 = 6.6 \times 10^{-3}$ min.$^{-1}$, calculated by the procedure elaborated in the Experimental. By analogy with the reaction sequence in BENCAA hydrolysis, k_1 would represent the specific rate of cyclic imide formation, and k_2 the specific rate of imide hydrolysis. Since, in this case the imide intermediate would be N-substituted, proton production should occur only in step 2. Actually, the specific rate of proton production, derived from alkali uptake experiments (see Fig. 2), was similar to the specific rate k_2 derived from the optical rotation measurements. The numerically larger constant k_1 quantitatively accounts for the observed "lag" in proton production, as shown in Fig. 2. By the above-mentioned methods, both specific rates (k_1 and k_2) could be studied as a function of pH. Results in dioxane–0.4 M aqueous NaCl (1:1 v./v.) at 25° are listed in Table II. The results show that both specific rates are first-order in [OH$^-$] over the pH range examined.

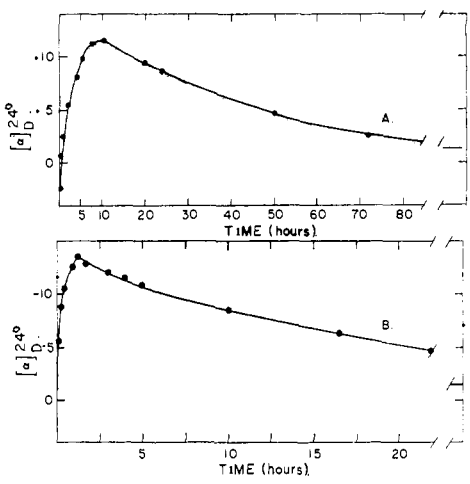


Fig. 7.—Changes in optical rotation at 589 mμ during the course of hydrolysis of BENCAA at pH 10.5 (A) and BENCAMA at pH 9.0 (B) in dioxane–0.5 M borate buffer (1:1 v./v.) at 24°.

The effect of solvent composition on the rate of hydrolysis of BENCASA was also studied. In the dioxane–water mixtures employed the measured pH (glass electrode) always corresponded to $-\log a_{H^+}$.[47,48] This quantity was fixed at 10 (pH 10) in all experiments involving the effect of solvent composition on reaction rate. Results are given in Table III.

(47) H. P. Marshall and E. Grunwald, *J. Chem. Phys.*, **21**, 2143 (1953).

(48) T. Inagami and J. M. Sturtevant, *Biochim. Biophys. Acta*, **38**, 64 (1960).

TABLE II

FIRST-ORDER SPECIFIC RATES IN THE REACTIONS OF β-BENZYL ESTERS OF ASPARTYL-SERYL DERIVATIVES IN DIOXANE–0.4 *M* AQUEOUS NaCl (1:1 v./v.) AT 25°

	L-BENCASA		D-BENCASA	BENCASME		ETACASA
*p*H	k_1[a]	k_2[a]	k_2[a]	k_1[a]	k_2[a]	k_2[a]
8.00	0.795					
8.25	1.60					
8.52	2.22					
8.64	2.72					
9.00	6.20	0.87	0.855	3.79	0.458	
9.50		1.95	3.63			2.00
10.0		7.18	9.50		3.05	4.16
10.50		52.7	47.3			17.60
11.00		82.7	78.2			

[a] In min.$^{-1}$ × 10^2; k_1 refers to imide formation, k_2 to imide hydrolysis.

TABLE III

FIRST AND SECOND-ORDER SPECIFIC RATES OF BENCASA HYDROLYSIS AS A FUNCTION OF SOLVENT COMPOSITION AT *p*H 10.0, 24°

Solvent compn. dioxane–water (v./v.)	Dielectric constant	$k_2 \times 10^2$, min.$^{-1}$	[OH$^-$], $M \times 10^6$	$k_2/[OH^-] \times 10^{-4}$, M^{-1} min.$^{-1}$
7:3	18	1.4	0.015	93
1:1	34	6.4	0.75	8.5
1:2	49	32	6.0	5.3
1:3	56	41	14	2.9
1:4	61	43	24	1.8

Since the ion product of water, K_w, is dependent on the solvent composition, it was necessary to estimate the hydroxyl ion concentration from the measured *p*H in each solvent, by graphical interpolation of the data presented by Harned and Owen[49] on the numerical values of K_w in various dioxane–water mixtures. At the *p*H's employed in these studies, the mean activity coefficients of H^+ and OH^- are near unity. The values of hydroxyl-ion concentration cited in Table III are precise to ±5%. The dielectric constants (see Table III) of these mixtures were interpolated from the data presented by Harned and Owen.[50]

β-Benzyl-N-carbobenzyloxy-L-aspartylmethyl Amide (BENCAMA).—The specific rates of hydrolysis of BENCAMA at several *p*H values in dioxane–water (1:2 v./v.) are listed in Table I. A sample hydrolysis curve at *p*H 10 in the above solvent is shown in Fig. 3. Following completion of the hydrolysis reaction in the above-mentioned solvent at *p*H 11, the reaction mixture was acidified to *p*H 2.5 and a potentiometric titration with alkali was carried out. One equivalent of acid with pK_a 4.5 was found. To determine the chemical composition of the hydrolyzate obtained on exposure of BENCAMA to *p*H 10.5 for 48 hr. at 24°, the analytical procedures given for the case of BENCAA were employed. A molar ratio of N-methylasparagine to N-methylisoasparagine of 6:4 was determined from the course of CO_2 evolution on treatment with N-bromosuccinimide of the catalytically hydrogenated hydrolysate. Paper electrophoresis of the decarbobenzyloxylated hydrolyzate in 0.05 *M* veronal buffer, *p*H 8, gave two spots of almost equal intensity on spraying with ninhydrin. One was electrophoretically similar to asparagine and the other was similar to isoasparagine.

The change in optical rotation during the course of ester hydrolysis of BENCAMA in dioxane–borate buffer (1:1 v./v.) at *p*H 9.5 is given in Fig. 7, curve B. The buffer used maintained a constant *p*H at early stages of the reaction (until the maximum in optical rotation was reached). At later stages, the *p*H dropped (see Experimental). Hence, only the specific rate, k_1, could be precisely determined from polarimetric data. The specific rate, k_2, was determined from the *p*H-stat rate data. The optical rotation–time curve in the region of the maximum of optical rotation (Fig. 7, curve B) was found to be consistent with the value of k_2 derived from the *p*H-Stat data. The values of k_2 and k_1/k_2 are given in Tables I and IV.

TABLE IV

RATIO OF SPECIFIC RATES FROM POLARIMETRIC DATA AT 24°

Compound	*p*H	Dioxane–buffer (v./v.)	k_1/k_2
BENCAA[c]	11	1:2[a]	140[e]
BENCAMA[c]	9.5	1:1[a]	40
BENCAEA[c]	9.5	1:2[a]	20
	9.0	2:3[a]	15
BENCAGA[c]	8.8	1:2[a]	30
BENCASA[d]	8.8	2:3[a]	25
	9.0	1:1[b]	10
	9.0	7:3[b]	15
BENCASME[d]	9.0	1:1[b]	8.3

[a] In 0.5 *M* borate buffer. [b] In tris buffer (μ = 0.2); the ratio at 1:1 dioxane buffer is based on the averaged values for the k's obtained from a *p*H *vs.* log k plot. [c] 25 mg./ml. [d] 10 mg./ml. [e] k_2 is uncorrected (*cf.* Table I, footnote *c*).

β-Benzyl-N-carbobenzyloxy-L-aspartylethanol Amide (BENCAEA).—The rate of hydrolysis of BENCAEA in dioxane–water (1:2 v./v.) at *p*H 10.0 is illustrated by Fig. 3. The reaction, in this solvent, has been studied as a function of *p*H. The results are summarized in Table I. The potentiometric titration with alkali of the acidified (*p*H 2.5) hydrolysis product resulted in a curve similar to that of Fig. 5 (CASA). The change in optical rotation during the course of reaction was followed at two different dioxane–borate buffer compositions (3.5/6.5 and 2/3 v./v. at *p*H 9.5 and *p*H 9.0, respectively). From the results thus obtained, the values of k_1/k_2 given in Table IV were calculated (see Experimental).

β-Benzyl-N-carbobenzyloxy-L-aspartylglycyl Amide (BENCAGA).—The time course of hydrolysis of BENCAGA in dioxane–water (1:2 v./v.) at *p*H 10 is shown in Fig. 3. The hydrolysis rates in this solvent at various *p*H values are given in Table I. Acidification of the hydrolysis products followed by potentiometric titration resulted in a titration curve similar to that of the hydrolysis product of BENCASA. The pK_a of the product was 4.5. The rate constant ratio, k_1/k_2, derived from optical rotation data (as in Fig. 1) is given in Table IV.

(49) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," 2nd ed., Reinhold Publ. Corp., New York, N. Y., 1950, p. 581.

(50) Reference 49, p. 544.

N-Carbobenzyloxy-D-aspartyl-L-seryl Amide (D-BENCASA).—The rate of alkali consumption during the hydrolysis of D-BENCASA in dioxane–0.4 *M* aqueous NaCl (1:1 v./v.), 25°, over the *p*H range 9–11, was found to be nearly the same as that observed for L-BENCASA (see Table II). Optical rotatory changes during the course of the reaction were difficult to analyze due to the monotonic and small change in rotation observed. A less precise estimate of k_1 at *p*H 9.0 from a Guggenheim plot[51] over the early part of the reaction gave a value of $k_1 = 8.0 \times 10^{-2}$ min.$^{-1}$ in fair agreement with the value determined for L-BENCASA.

β-Benzyl-N-carbobenzyloxy-L-aspartyl-L-serine Methyl Ester (BENCASME).—The time-course of hydrolysis of BENCASME was followed both in the *p*H-Stat and in the polarimeter, at *p*H 9 and 10, at 25° in dioxane–0.4*M* aqueous NaCl (1:1 v./v.). The results (which are qualitatively similar to those obtained with BENCASA) are summarized in Tables II and IV.

β-Ethyl-N-acetyl-L-aspartyl-L-seryl Amide (ETACASA) was prepared to examine the role of other acyl and ester substituents on the rate of β-ester hydrolysis. Experiments are still in progress on this water-soluble derivative and will appear in a future communication (S.A.B. and J.H.C.). For comparison with the above studies, the hydrolysis reaction was followed both in the *p*H-Stat and in the polarimeter in dioxane–0.4 *M* aqueous NaCl (1:1 v./v.). The results obtained yielded the specific rates given in Table II.

Discussion

A. Comparison of the Reaction Rate Constants.—The β-benzyl esters listed in Table I differ widely in their specific rates of hydrolysis (by factors of as much as 2×10^3). The rates of alkaline hydrolysis of esters of unsubstituted aliphatic acids, on the other hand, fall within a narrow range. Thus ethyl propionate in acetone–water (7:3 v./v.) and benzyl acetate in acetone–water (6:4 v./v.), (dielectric constants $D = 39$ and 44, respectively) have second-order specific rates of alkaline hydrolysis of 1.3 min.$^{-1}M^{-1}$ and 4.2 min.$^{-1}$ M^{-1}.[52] The second-order specific rate of hydrolysis (k_2/[OH]) of BENCASA in dioxane–water (7:3 v./v.; $D = 18$) is 9.3×10^5 min.$^{-1}$ M^{-1} (Table III), a factor of at least 2.3×10^5 greater than the rate of above-mentioned alkaline aliphatic ester hydrolysis. Moreover, the rates of hydrolysis of aliphatic esters are known to decrease with decreasing dielectric constant (increasing fraction of organic solvent).[53,54] Thus a comparison of the rates of hydrolysis of aliphatic esters with the peptide esters reported (Table I) at the same low value of D would result in a still larger difference in rate. In the case of BENCASA the second-order specific rate constant k_1/[OH$^-$] (see Tables III and IV), reflecting the rate of debenzylation, is nearly 10^7 times greater than the second-order specific rate of alkaline hydrolysis of ethyl propionate in dioxane–water (7:3 v./v.). These increased rates must reflect unusual structural features of the BENCASA molecule.

B. A Proposed Mechanism for the Hydrolysis of β-Esters of Aspartyl Peptides.—From our results with the β-benzyl esters of aspartyl peptides we have arrived at the general mechanism shown in Fig. 8.

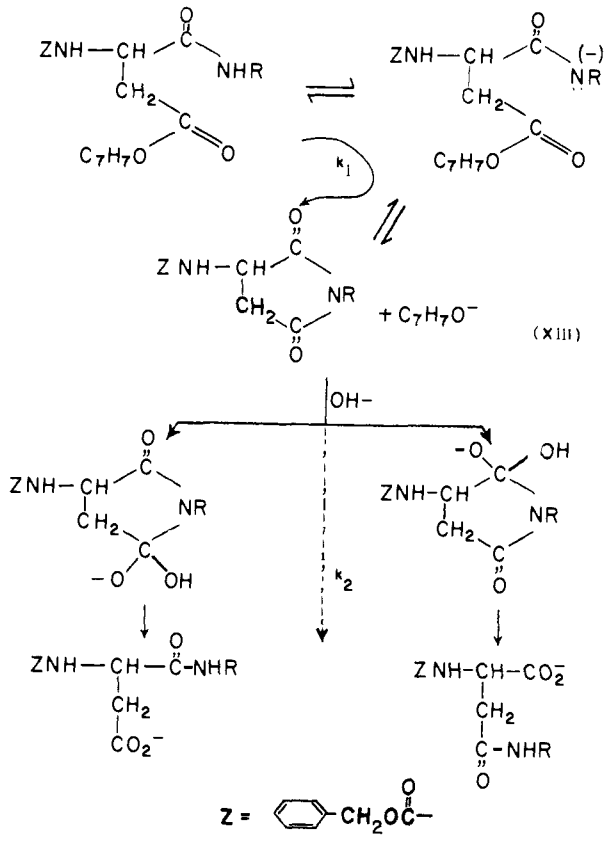


Fig. 8.—General mechanism for hydrolysis. The reversal of imide formation by benzylate (upper right) should yield the α-ester isomer in addition to the β-isomer illustrated.

The first stage in the complex reaction path of Fig. 8 is the reversible dissociation of a proton from the nitrogen of the α-aspartyl peptide (amide) linkage. This is followed by displacement of the alcoholate of the β-carboxyl by the negative amidate ion to form the five-membered succinimide, with elimination of alkoxide ion. The succinimide subsequently undergoes nucleophilic addition or displacement with hydroxyl ion (see discussion below) at either carbonyl carbon resulting in the opening of the ring to either an α- or a β-peptide.

The mechanistic pathway (Fig. 8) is dictated by the following experimental facts.

(1) The corresponding five-membered succinimide derivative was isolated and identified in two cases: In the reactions of BENCAA and BENCAMA, intermediates with specific rotations of $[\alpha]^{20}$D −39° and −35°, respectively, could be isolated. The intermediate from BENCAA was identified as N-carbobenzyloxy-L-aminosuccinimide.[40] The

(51) E. A. Guggenheim, *Phil. Mag.*, **9**, 538 (1926).

(52) "Tables of Chemical Kinetics," U. S. Natl. Bureau of Standards Circular No. 510, Washington, D. C., 1951, pp. 104, 102.

(53) E. Tommila, A. Koivisto, J. P. Lyrra, K. Antell and S. Heimo, *Ann. Acad. Sci. Fenn., Ser. A, Chem.*, **47** (1952).

(54) (a) E. Tommila, *Suomen Kem.*, **B25**, 37 (1952); (b) P. Madhaven Nair and S. V. Anantakrishnam, *Proc. Indian Acad. Sci.*, **32A**, 187 (1950).

elemental analysis of the intermediate from BENCAMA was that expected for carbobenzyloxyamino (N-methyl)-succinimide.

(2) The imide ring was implicated as an intermediate: The large negative optical rotations exhibited by the imides formed during the hydrolysis of BENCAA and BENCAMA are absent in both the reactant and the products. Similarly, intermediates with this property of large negative optical rotation have been observed as transients during the hydrolysis of all other aspartyl peptides containing an ionizable hydrogen on the peptide nitrogen (see Figs. 1, 7 and Table IV).

(3) Dissociation of the hydrogen from the nitrogen of the peptide bond was essential for rapid reaction rates: The dimethyl amide BENCADMA, which has no ionizable hydrogen at the nitrogen of the peptide bond, shows a slow rate of benzyl ester hydrolysis (see Table I). The rate of hydrolysis of N-carbobenzyloxy-β-alanine benzyl ester is also slow (see Table I).

(4) The hydrolysis of the imide was demonstrated to be the slow step in the over-all reaction: The alkaline hydrolysis of carbobenzyloxyamino-(N-methyl)-succinimide, prepared from BENCAMA, had a specific rate equal to that for the over-all hydrolysis of BENCAMA.

(5) The two-step mechanism given in Fig. 8 is quantitatively in accord with the kinetics of proton production: Detailed examination of the rate of proton production (alkali consumption) during the course of hydrolysis of BENCASA (Fig. 2) revealed a "lag," in accord both with the ratio of the two rate constants (Table IV) and the two-step mechanism proposed (Fig. 8). Only the second step of the reaction (imide hydrolysis), by virtue of the ionization of the carboxylic acid in the product at these *p*H values, involves a stoichiometric uptake of alkali.

(6) Both aspartyl (α-)peptides and isoaspartyl (β-)peptides have been identified as hydrolysis products: Removal of carbobenzyloxy groups from the hydrolysis products of BENCAA, BENCAMA and BENCASA revealed upon paper electrophoresis a mixture of α- and β-peptides.

C. Structural Factors Influencing the Rates of Formation and Hydrolysis of Imides.—That imides are readily formed from esters of asparagine and isoasparagine,[40] and from β-esters of aspartyl peptides[55] has already been clearly established. The rapid alkaline hydrolysis of imides was first demonstrated by Titherley and Stubbs.[56] More recently Edward and Terry[57] have measured the rate of alkaline hydrolysis of succinimide in aqueous solution. They postulated that only the undissociated imide (Imide^0) appeared in the rate equation

$$v = k[\text{imide}^0][\text{OH}^-]$$

The second-order specific rate of succinimide hydrolysis in aqueous solution at 25° (k = 420 min.$^{-1}$ M^{-1}) is very large as compared to that of esters under similar conditions (*e.g.*, k = 2.1 min.$^{-1}$ M^{-1} for ethyl propionate[51]). As expected, it is similar to the second-order specific rate of hydrolysis of the imide intermediate prepared from BENCAMA ($k = k_2/[\text{OH}^-]$ = 380 min.$^{-1}$ M^{-1}; see Tables III and V) and of the imide derived from BENCAA ($k = k_2/[\text{OH}^-]$ = 820 min.$^{-1}$ M^{-1}; see Tables III and V, calculated for the un-ionized form).

TABLE V

HYDROLYSIS RATE AS A FUNCTION OF THE ACID STRENGTH OF THE TERMINAL AMINO SUBSTITUENT

β-Benzyl-N-carbobenzyloxy-aspartyl derivative	Corresponding terminal acid	pK_a	$k_2 \times 10^2$, min.$^{-1}$; *p*H 10.0, dioxane-water (1:2 v./v.) 24°
BENCAA	NH_4^+	9.25	0.49[a]
BENCAMA	$CH_3NH_3^+$	10.4	0.23
BENCAEA	$NH_3CH_2CH_2OH^+$	9.44	1.15
BENCAGA	$NH_3CH_2CONH_2^+$	7.8	7.0
BENCASA	$NH_3CH(CH_2OH)CONH_2^+$	7.37	32, 7.0[b]
BENCASME	$NH_3CH(CH_2OH)CO_2CH_3^+$	7.18	3.0[b]

[a] Value corrected according to the procedure given in Table I, footnote *c*. [b] In dioxane–0.4 M aqueous NaCl (1:1 v./v.), 25°.

The rate of ring closure to form succinimide derivatives has not been previously studied except for the case of unsubstituted succinamide,[58] although the facility with which this reaction occurs has been indicated. Battersby and Robinson[55] have reported the formation of imide from N-benzoylisoasparagine ethyl ester and one equivalent of base (NaOH or Na_2CO_3). Excess of base led to the formation of carboxylic acids. These authors also found that the β-ester of N-benzoylaspartyl-α-glycyl-N-hexylamide was hydrolyzed readily in the presence of excess base, yielding a mixture of α- and β-peptides. The same mixture was obtained with the α-ester of the corresponding β-peptide as the starting material. This result indicates that the hydrolysis proceeded in both cases *via* the same cyclic imide intermediate.

The optical rotatory changes associated with imide formation presented a novel approach for following the formation and disappearance of the cyclic intermediates. By the kinetic methods described above (Fig. 1) it was possible to analyze the effect of peptide structure on the hydrolytic reaction rate in terms of the two constants, k_1 (imide formation) and k_2 (imide hydrolysis).[59]

For the various β-benzyl aspartate derivatives tested, with the exception of BENCAA where electrostatic effects on k_2 are of major importance, the ratios k_1/k_2 vary by a factor of less than five (see Table IV). The large differences in k_2 as well as in k_1 for the compounds listed in Tables I and IV seem therefore to originate in structural characteristics which affect ring closure and ring

(55) A. R. Battersby and J. C. Robinson, *J. Chem. Soc.*, 259 (1955).
(56) A. W. Titherley and L. Stubbs, *ibid.*, **105**, 299 (1914).
(57) J. T. Edward and K. A. Terry, *ibid.*, 3527 (1957).
(58) M. B. Vigneron, P. Crooy, F. Kezdy and A. Bruylants, *Bull. soc. chim. Belg.*, **69**, 616 (1960).
(59) Recently we have observed spectroscopically the appearance of an intermediate in the 240 mμ region. The time constants derived from the spectroscopic data are in accord with those obtained polarimetrically; the spectral change is a maximum in the same wave length region as that reported for the succinamide → succinimide reaction (ref. 58).

hydrolysis similarly. In the elucidation of these structural factors, the following findings should be considered: (1) The most rapid rate of hydrolysis measured (that for BENCASA) involves a substrate with three peptide bonds and a β-hydroxymethyl group. (2) The diastereoisomer β-benzyl-N-carbobenzyloxy-D-aspartyl-L-seryl amide is hydrolyzed at the same rate as the L-isomer. (3) Rates intermediate to BENCAMA and BENCASA are found in compounds containing (a) a β-hydroxymethyl group and two peptide bonds (BENCAEA), (b) three peptide bonds (BENCAGA), or (c) a β-hydroxymethyl group, two peptide bonds and a terminal methyl ester (BENCASME). (4) The rate of hydrolysis of a compound in which carbobenzyloxy and β-benzyl have been replaced by acetyl and β-ethyl, respectively (ETACASA), is nearly as rapid as the rate of hydrolysis of BENCASA.

D. The Effect of *p*H and Dielectric Constant on the Cyclization and Hydrolysis Rates of BENCASA.—The hydrolysis of BENCASA, because of its very rapid rate and its potential biological interest, was studied in more detail than that of the other compounds. From optical rotation–time curves (as in Fig. 1) and from the *p*H-Stat data, the specific rate of formation (k_1) and hydrolysis (k_2) of the intermediate was determined at various *p*H. Both these specific rates are first order in hydroxyl ion concentration. The optical rotation–time curves during the reaction of BENCASA were the same at all *p*H values measured except for the time scale. Any deviation from first-order dependence of either k_1 or k_2 on hydroxyl ion would result in a different rotation maximum, a change in the shape of the curve and a different value for k_1/k_2. From the value of the optical rotation at the maximum (Fig. 1) and the values of k_1 and k_2, the *molar* rotation of the intermediate could be calculated. The molar rotation $\phi^{25}_{436m\mu}$ is $-20°$ (*c* 1 in 1:1 v./v. dioxane–water), twice the value observed with the imides prepared from BENCAMA. The molar rotations of both the reactant and the products of hydrolysis of BENCASA are essentially the same as those obtained for all other N-carbobenzyloxy-L-aspartyl amides studied.

The effect of solvent composition on the rate of hydrolysis of BENCASA was studied. As has already been mentioned above, the rate of BENCASA hydrolysis is first order in hydroxyl ion concentration. If the reaction rate is measured at constant *p*H as a function of solvent composition, the change in K_w and hence hydroxyl ion concentration must be taken into account in order to calculate the second-order specific rate. The second-order specific rate thus calculated *increases* with *increasing* percentage of dioxane. A plot of log $k_2/[OH^-]$ *vs.* $1/D$ (where D is the dielectric constant) in this solvent system is shown in Fig. 9. The plot is nearly linear with a large positive slope ($m = +40$). In contrast, the alkaline or acid hydrolysis of esters[53,54,60] in water–alcohols, water–acetone and water–dioxane shows both *negative* and smaller slopes (the slope is especially small in dioxane–water). The alkaline and acid hydrolysis of amides[61a] and the alkaline hydrolysis of lactones[61b] also give negative slopes in such plots. The second-order specific rates of alkaline hydrolysis of BENCAEA at two widely different dioxane–water compositions (1:2 and 7:3) were in approximately the same ratio as those obtained for BENCASA (see Fig. 9).

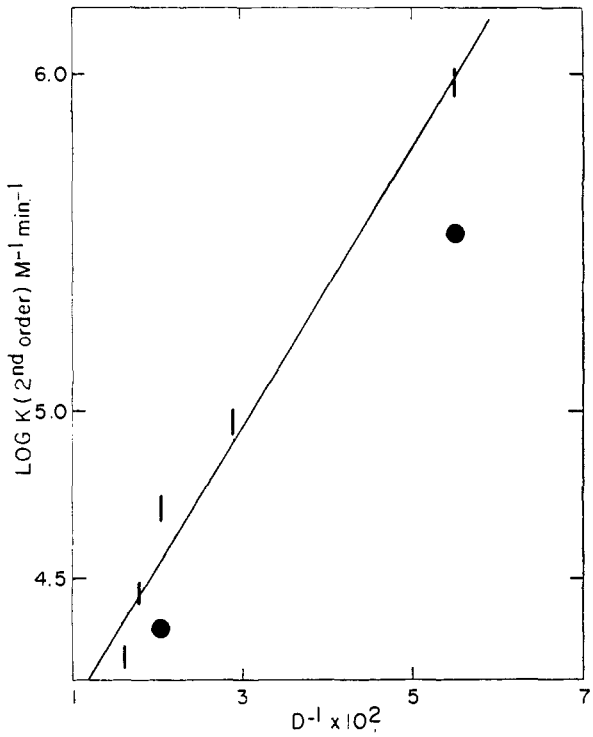


Fig. 9.—Variation of the second-order specific rate of hydrolysis ($k = k_2/[OH^-]$) as a function of the bulk dielectric constant of dioxane–water mixtures at 24°. Rectangles refer to log *k* for L-BENCASA, circles to 1 + log *k* for BENCAEA.

In order to investigate the effect of dielectric constant and/or solvent composition on k_1, the optical rotatory changes accompanying the hydrolysis of BENCASA were followed at *p*H 9.15 in 7:3 v./v. dioxane–water ($D = 18$), where $k_2/[OH^-]$ is more than ten times its value in 1:1 (v./v.) dioxane–water ($D = 34$). From the results of Tables III and IV, it is clear that both k_1 and k_2 are similarly affected by dioxane concentration. Thus, variations in substrate structure and solvent composition, although exerting a profound influence on individual specific rates, have little influence on the ratio k_1/k_2 for the aspartyl peptides studied. A possible explanation for this uniform effect on the two rates might involve a strong inductive effect from the terminal carboxamido group (BENCAGA) or hydroxymethyl group (BENCAEA) on both ring closure and imide hydrolysis (see discussion below).

E. Further Analysis of the Reaction Mechanism.—Evaluating the findings discussed in sections C and D, namely the effects of substituent structure and dielectric constant of the medium on the reaction rates, an attempt is made in the following to add more details to the mechanism described in section B.

(60) J. E. Quinlan and E. S. Amis, *J. Am. Chem. Soc.*, **77**, 4187, (1955).

(61) (a) K. J. Laidler and P. A. Landskroener, *Trans. Faraday Soc.* **52**, 200 (1956); (b) E. Tommila and M. P. O. Ilomäki, *Acta Chem. Scand.*, **6**, 1249 (1952).

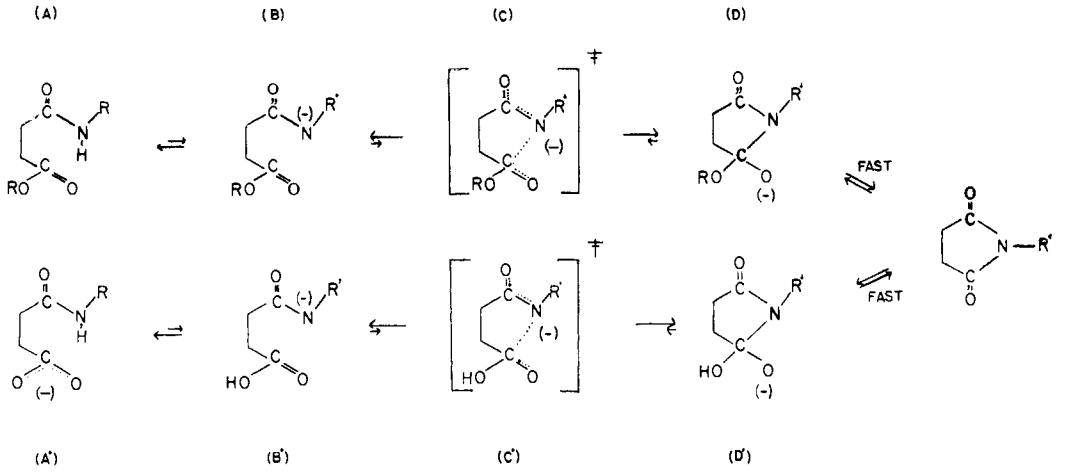
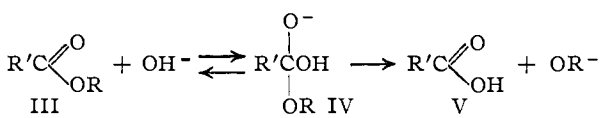


Fig. 10.

From the dependence of reaction rates on dielectric constant, particularly from the sign of the slope d (log K)/d(D^{-1}) (see Fig. 9), it should be possible to draw certain conclusions regarding the rate-controlling process in a reaction involving ions and dipoles. However, due to the lack of a completely satisfactory theory[61b,62] of the effect of dielectric constants[63] on reaction rates, such conclusions are necessarily of a qualitative nature.

As regards alkaline hydrolysis of esters, the generally assumed reaction path[3] involves nucleophilic attack by hydroxyl ion with the formation of a negatively charged intermediate (IV) followed by elimination of the alkoxide ion. The second-order specific rate of this reaction increases with increasing dielectric constant (except at very low concentrations of water[52]).



The effect of change in dielectric constant on the rate of formation and alkaline hydrolysis of the imides reported here is opposite to that in the case of esters, amides and lactones discussed above. Moreover, the magnitude of the slope of the line in Fig. 9 is very large relative to that usually encountered in the hydrolysis of carboxylic acid derivatives. This slope, d (log k)/d($1/D$), is equal to 50, as compared to values of about -10 for the hydrolysis of aliphatic esters[53,54] and values of about $+100$ for the second-order reactions of oppositely charged monovalent ions. Whatever the mechanism of imide formation and imide hydrolysis is, it must involve a transition state of significantly different electrostatic character from that encountered in aliphatic ester and amide hydrolysis. An explanation for this behavior may be found in the following scheme (Fig. 10).

(62) See for example E. S. Amis, *J. Chem. Educ.*, **30**, 351 (1953); E. S. Amis and G. Jaffe, *J. Chem. Phys.*, **10**, 598 (1942); K. J. Laidler, "Chemical Kinetics," McGraw-Hill Book Co., Inc., New York, N. Y., 1950, Chapt. V; E. S. Amis, "Kinetics of Chemical Change in Solution," The Macmillan Co., New York, N. Y., 1949, Chapt. VIII.

(63) The difficulties of using bulk dielectric constants in a microscopic situation has been treated theoretically by J. G. Kirkwood and F. H. Westheimer, *J. Chem. Phys.*, **6**, 506 (1938).

The position of the transition states C and C', in the assumed reaction path, accounts for the observed dependence of the specific rates k_1/[OH$^-$] and k_2/[OH$^-$] on changes in the dielectric constant of the medium. The scheme suggested is also in accord with the experimental findings that particular aspartyl peptide substituents influence (*via* inductive effects) both specific rate constants in a similar manner.

The salient features of the above scheme are: (1) The rate-controlling steps in both formation of imide and its hydrolysis of esterification involve the formation of the peptide nitrogen–β-carbonyl carbon bond *without* simultaneous cleavage of the β-carbonyl C–OR bond. (2) The transition state (C or C') is a negatively charged species in which the negative charge is distributed amongst the two carbonyl oxygens, and the central (imide) nitrogen, whereas in all four ionic reactants and products (B, B', D, D') the charge is more localized. This "smearing" of the charge in the transition state makes the transition state more stable relative to the negatively charged reactants and hence the reaction rate *increases* in solvents of low dielectric constant. (3) The rates of association and dissociation of hydroxyl or alkoxyl to the imide must be fast relative to the rate of C–N bond formation or rupture. If this were not the case, the effect of dielectric constant on imide hydrolysis would be the same as on ester (or amide or γ-lactone) hydrolysis. (4) The ability of the substituent, R', to withdraw electrons will govern the relative concentrations of the ion B and the tetrahedral intermediate D', and hence the rates of imide formation and hydrolysis. Both these rates will be affected by the nature of R' (by the "inductive" effect of R') in the same manner.

The large inductive effect of terminal groups (carboxamido, β-hydroxymethyl) in the above peptide sequences may account for enhanced rates of both ring closure and hydrolysis. The same electron-withdrawing properties of the carboxamido group which make glycyl amide a weaker base than methylamine or ammonia (see Table V) would facilitate ionization at the peptide nitrogen (and hence ring closure) more readily in BENCAGA than in BENCAMA or BENCAA. Similarly

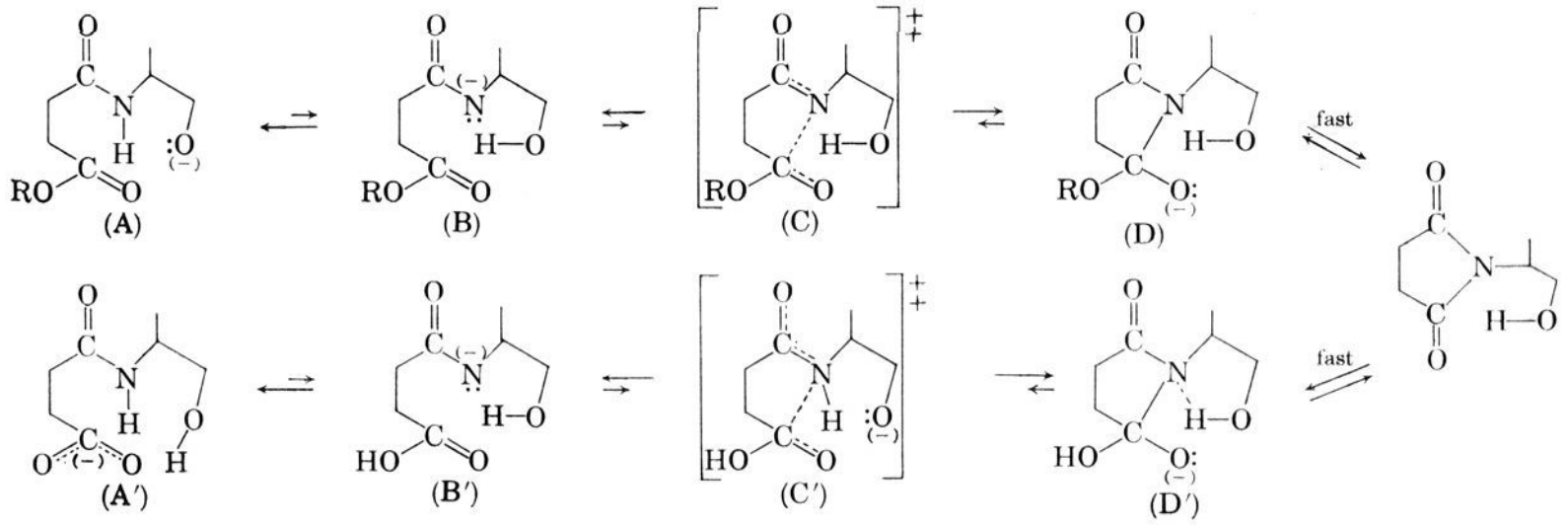


Fig. 11.

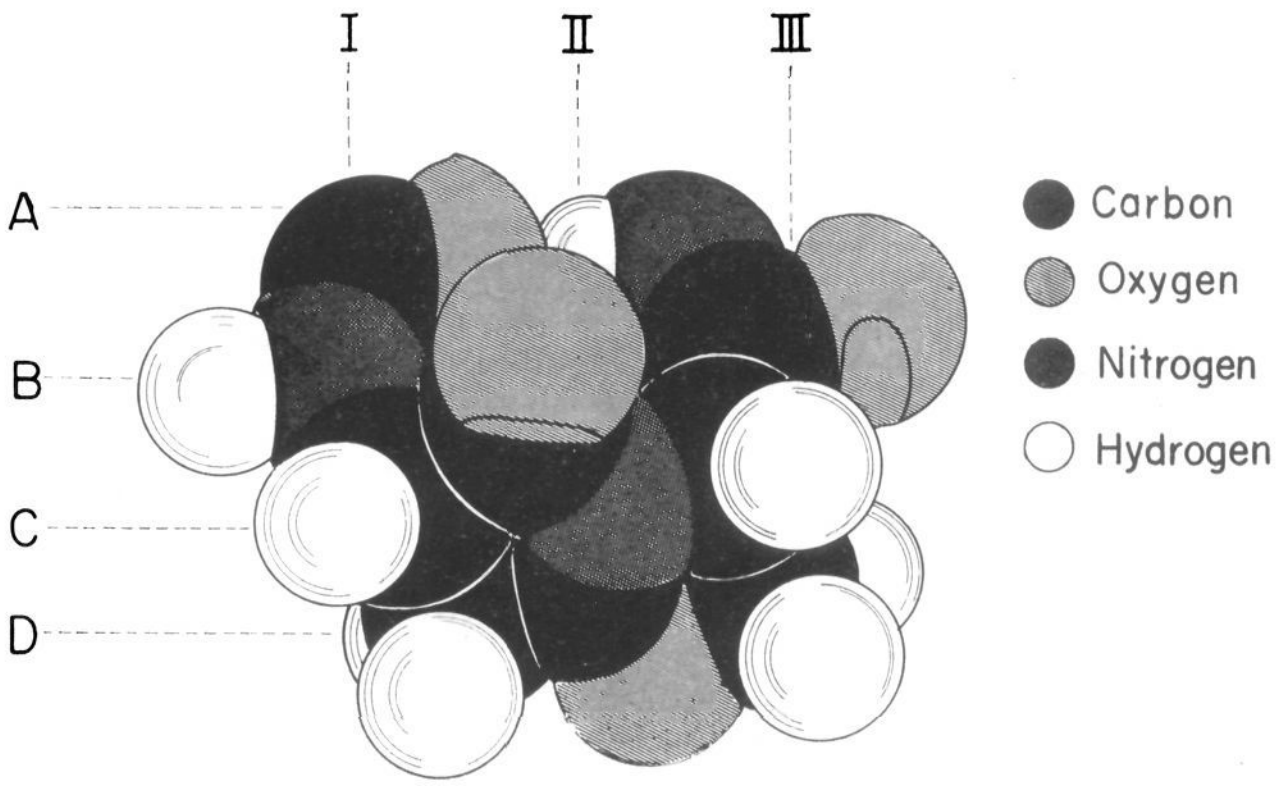
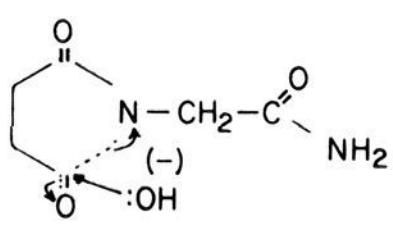


Fig. 12.—Molecular model of compound VIII.

imide ring BI—BII / DI—DII BII—BIII / DII—DIII oxazolidine ring

AII, hydrogen bond; CII, imide nitrogen; DII, β-carbon of aspartic-oxygen of serine; DII, behind plane, oxygen of the β-carbonyl (not shown).

in hydrolysis of the imide, the leaving group $-\overset{(-)}{N}-CH_2CONH_2$ in VI would be stabilized relative to the $-\overset{(-)}{N}-CH_3$ or unsubstituted leaving group.



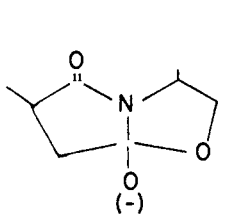
(VI)

The relative rates of ring closure and hydrolysis of aspartyl peptides should therefore be directly related to the acid strengths of the corresponding amines. According to this mechanism, the stronger the acid, the more rapid should be the reaction rate. A comparison of hydrolytic rate with acid strength is given in Table V. Although the rates agree qualitatively with the mechanism proposed above, there are quantitative inconsistencies both in the very rapid rate of alkaline hydrolysis of BENCAEA relative to the rate of hydrolysis of the electrically neutral species of imide from BENCAA and in the rapid rate of hydrolysis (k_2) of BENCASA relative to BENCASME. The difference between k_1 in BENCASA and in BENCASME is even greater (*i.e.*, more inconsistent with a purely inductive mechanism). It appears, therefore, that although the rates of both cyclization and hydrolysis are related to the acid strength of the peptide nitrogen, factors other than this inductive effect are involved as well.

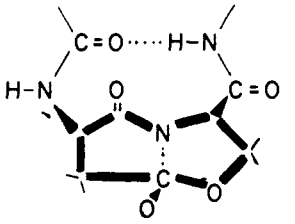
The largest additional structural effect revealed in Table V is that due to the presence of a β-hydroxymethyl group (compare BENCAEA and BENCASA with BENCAMA and BENCAGA, respectively). The β-hydroxyl of serine has been

shown to act as a reactive nucleophile[24] due to its unusually high dissociation constant.[64] This high dissociation constant may also account for the intramolecular catalysis observed both with respect to ring closure and to ring opening. In the former case, the serinate ion would facilitate the ionization of the peptide nitrogen (A → B) by abstraction of the proton (general base catalysis) as shown in the following scheme (Fig. 11). In the latter case the β-hydroxyl would supply a proton to the ring nitrogen of the transition complex C′ (general acid catalysis), thus facilitating ring opening and stabilizing B′ relatively to C′. The structures A, B, C, D, A′, B′, C′ and D′ of Fig. 11 correspond to the respective structures of Fig. 10.

F. Speculation on the Chemistry of Proteolytic Enzyme Sites.—The unusual reactivity of the serine hydroxyl at the catalytic site of some proteolytic enzymes suggests the possibility that the hydroxyl group of serine in the sequence -Gly-Asp-Ser-Gly- is somehow chemically altered. One possibility of such a covalently altered structure, involving both the aspartic acid and the serine residue of the active site sequence, is the bicyclic structure VII. This structure, which is the intramolecular analog of structure D (Fig. 10), could be rapidly formed by a nucleophilic attack of the serine hydroxyl on the β-carbonyl of an existing imide ring, possibly aided by the presence of a general base catalyst such as imidazole. It might be established by hydrogen bonding between the carbonyl oxygen of the amino acid preceding the aspartyl residue and the peptide nitrogen of the residue following the serine, as indicated in VIII. In fact, a compact structure on the basis of VIII can be constructed from "space filling (Cal. Tech.) atoms" in which all the peptide bonds are coplanar and in the "*trans*" configuration (Fig. 12).

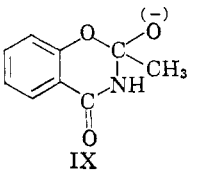


VII

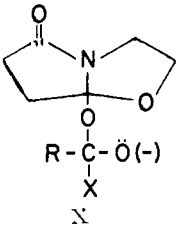


VIII

A bicyclic intermediate, IX (chemically related to VII), involved in acyl transfer reactions has been proposed by Brenner[32] for the reversible O–N isomerization of *O*-acetylsalicylamide to N-acetylsalicylimide.



IX



X

The nucleophilic oxygen at the bridgehead carbon atom in structure VIII could serve as a nucleophile in enzymic catalysis[66,67] and the conventional "Michaelis–Menten (enzyme–substrate) complex" could involve chemical bonding to substrate without concomitant breaking of any of the substrate bonds as in X.[66,67]

Although the above-mentioned mechanism of catalysis must be considered highly speculative at this time, it does suggest the possibility of treating enzyme reaction mechanisms in terms of the primary sequence of amino acids in the protein molecule, taking into consideration the possible existence of unconventional covalent structures of high reactivity. Two such possible structures, one involving arginine and aspartic acid[68] and the other involving serine oxazoline and aspartic acid,[34] have been recently described.

Acknowledgment.—This work was supported by a grant (B-5887) from the National Science Foundation, and a grant (A-3083) from the National Institutes of Health.

(64) T. C. Bruice, T. H. Fife, J. J. Bruno and N. E. Brandon, *J. Am. Chem. Soc.*, **84**, 1973 (1962).

(66) S. A. Bernhard, *J. Mol. Biol.*, in press (1962).

(67) S. A. Bernhard, *J. Cell. Compt. Phys.*, **54** (suppl. 1), 195 (1959).

(68) B. F. Erlanger, *Proc. Natl. Acad. Sci.*, **46**, 1430 (1960).

[CONTRIBUTION FROM THE HORMONE RESEARCH LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY 4, CALIF.]

Preparation of L-Lysyl-L-lysyl-L-arginyl-L-arginyl-L-proline

BY JOHANNES MEIENHOFER AND CHOH HAO LI

RECEIVED NOVEMBER 18, 1961

The pentapeptide L-lysyl-L-lysyl-L-aringyl-L-arginyl-L-proline has been prepared. The optical purity of the product was established by microbiological assay of the amino acid content of the acid hydrolyzate of the peptide as well as by enzymic digestion. It was further demonstrated that the arginyl-proline bond in the pentapeptide is resistant to the action of leucine aminopeptidase and trypsin.

Introduction

In the course of synthetic work on a peptide sequence occurring in adrenocorticotropin (ACTH), the protected pentapeptide intermediate N^{α}-carbobenzoxy-N^{ϵ}-tosyl-L-lysyl-N^{ϵ}-tosyl-L-lysyl-N^{G}-tosyl-L-arginyl-N^{G}-tosyl-L-arginyl-L-proline had been synthesized.[1] It occurred to us that it would be of interest to prepare the free peptide, which contains an unusual sequence of amino acid residues. In addition, it would afford an opportunity to examine the optical purity of the constituent amino acids in the free pentapeptide.

(1) C. H. Li, J. Meienhofer, E. Schnabel, D. Chung, T. B. Lo and J. Ramachandran, *J. Am. Chem. Soc.*, **82**, 5760 (1960); **83**, 4449 (1961).