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Kinetic Studies on the Alkali-Catalyzed Hydrolysis and Epimerization of Model Alkyl and Hydroxyalkyl Di- and Tripeptides†

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ABSTRACT: The present work shows that the alkaline stability of the amide bond in alkyl and hydroxyalkyl dipeptides is a function of the size and the position of the α and α' substituents. When the various alkyl substituents are listed according to their stabilizing effects, the following order is obtained: $(CH_3)_2CHCH_2 \geq (CH_3)_2CH > CH_3CH_2 > CH_3 > H$. The relative alkaline stability of serine- and threonine-containing dipeptides depends upon the position of the R″(HO)CH- group in $NH_2CH(R')CONHCH(R)COO^-$. In the R position, the hydroxyalkyl group facilitates hydrolysis, while in the R′ position, cleavage is impeded. Experimental evidence indicates that peptide bond hydrolysis in dilute alkali at constant ionic strength is strictly a function of a_{OH^-} and is not influenced by potential polyfunctional catalysts (*e.g.,* borate and phosphate). Plots of the pseudo-first-order rate constants (k_{obsd}) *vs.* K_w/a_{H^+} show that solvolysis plays no role in the cleavage of model dipeptides at pH values between 11.5 and 13.6. Based on the above findings, the relative rates of the alkali-catalyzed hydrolysis of each of the amide bonds in the tripeptides, L-Ala-L-Ser-Gly and Gly-L-Ser-L-Ala, can be predicted. In each case, the seryl residue has been found to labilize and to stabilize its own NH_2- and COOH-terminal bonds, respectively. In addition to the studies on hydrolysis, kinetic analyses have been made on alkali-catalyzed epimerization phenomena observed with seryl di- and tripeptides.

During a course of study related to the effect of alkali on the structure of rabbit muscle aldolase, it was found that upon exposure of both the native and the succinyl enzyme to pH 12.5 potassium borate over the temperature range 0–30°, selective peptide bond hydrolysis had occurred yielding a limited number of newly formed NH_2-terminal serine, threonine, and glycine residues (Sine and Hass, 1967, 1969). The invariant nature of the hydrolysis was reflected in the observation that no other amino-terminal residues were liberated after prolonged exposure to alkali. Additional work showed that the phenomenon observed with aldolase was equally applicable to other proteins, such as bovine serum albumin, lysozyme, and ribonuclease A (Hass *et al.,* 1968).

This study has been performed in an effort to gain further insight into the mechanism of selective peptide bond cleavage in proteins exposed to alkali.[1] In addition to investigating the influence of various α-alkyl and α-hydroxyalkyl substituents on the stability of several model di- and tripeptides, we have considered the catalytic effects of various buffers, hydroxide ion, and H_2O on amide bond cleavage. Emphasis has also been placed on investigating the kinetics of serine racemization in seryl peptides. The results derived from the latter studies have provided some understanding of serine's influence on the stability of protein primary structure at high pH values.

Materials and Methods

Materials. Glycylglycine was obtained from Calbiochem. L-Seryl-L-alanine and L-seryl-L-leucine were purchased from Miles Laboratories, Inc. All other dipeptides,

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[1] This report is an expansion of a preliminary communication by Jarboe *et al.* (1971).

except L-alanyl-L-serine and L-leucyl-L-serine, were purchased from Schwarz Mann. Chloromethylated polystyrene (1.2 mmol of Cl/g), *t*-BOC-*O*-benzyl-L-serine,[2] *t*-BOC-L-alanine, and *t*-BOC-glycine were also obtained from Schwarz Mann.

L-Alanyl-L-serine, L-leucyl-L-serine, glycyl-L-seryl-L-alanine, and L-alanyl-L-serylglycine were synthesized by the method of Merrifield (1969), using the procedures outlined by Stewart and Young (1969). The peptides were purified by ion exchange chromatography on a 100–200 mesh Dowex 50W-X4 (Bio-Rad Laboratories) column which had been equilibrated with pH 4.0 pyridine–acetate buffer (Schroeder, 1967). The column effluent was monitored for ninhydrin-positive material and appropriate fractions were pooled and lyophilized. The lyophilized compounds were crystallized from either aqueous ethanol or aqueous acetone. The following elemental analyses and melting points were obtained: L-alanyl-L-serine, mp 211–212° dec (Calcd for $C_6H_{12}O_4N_2$: C, 40.90; H, 6.87; N, 15.90. Found: C, 40.90; H, 7.04; N, 16.11); L-leucyl-L-serine, mp 211° dec (Calcd for $C_9H_{18}O_4N_2$: C, 49.53; H, 8.31; N, 12.83. Found: C, 49.44; H, 8.48; N, 12.65); L-alanyl-L-serylglycine, mp 199–200° dec (Calcd for $C_8H_{15}O_5N_3$: C, 41.20, H, 6.48; N, 18.02. Found: C, 41.49, H, 6.65; N, 18.23); and glycyl-L-seryl-L-alanine, mp 230° dec (Calcd for $C_8H_{15}O_5N_3$: C, 41.20; H, 6.48; N, 18.02. Found: C, 40.80; H, 6.63; N, 17.72).

All peptides were checked for both purity and amino acid content on the Beckman Model 120C automatic amino acid analyzer (Moore *et al.*, 1958).

Synthesis of D-*Seryl*-L-*leucine.* L-Seryl-L-leucine (450 mg in 45 ml of H_2O) was incubated at 90° with 450 ml of 0.03 M $KB(OH)_4$ (pH 12.6). After 24 hr, the solution was chilled in ice-water, neutralized with 6 *N* HCl, and dried by rotary evaporation. The resultant residue was acidified to pH 2.5 with HCl and was chromatographed on a 2.5 × 87 cm column of 100–200 mesh Dowex 50W-X4, according to the method of Schroeder (1967). The column was eluted with an increasing linear gradient formed from 2 l. each of 0.1 M pyridine–0.5 M acetic acid (pH 4.0), and 1.0 M pyridine–0.05 M acetic acid (pH 6.1). Fractions (15 ml) were analyzed for ninhydrin-positive material after spotting 10-μl aliquots on filter paper. Products were resolved in the following approximate elution volumes: serine, 300 ml; leucine, 600 ml; D-seryl-L-leucine, 950 ml; and L-seryl-L-leucine, 1300 ml. Appropriate fractions were combined and were dried by rotary evaporation to give 196 mg (44%) of the D,L dipeptide and 185 mg (41%) of the parent compound. Crystallization of D-seryl-L-leucine from aqueous ethanol gave colorless needles, mp 205–210° dec, $[\alpha]^{23}_{589}$ −47.9° (*c* 0.7, H_2O) (Calcd for $C_9H_{18}O_4N_2$: C, 49.53; H, 8.31; N, 12.83. Found: C, 49.34; H, 8.45; N, 12.80). The L,L isomer crystallized as shiny leaflets, mp 213–216° dec, $[\alpha]^{23}_{589}$ −13.9° (*c* 1.0, H_2O).

Determination of Rate Constants. Hydrolyses were conducted in a thermostated bath regulated to ±0.02°. Reaction vessels consisted of culture tubes closed with Teflon-lined screw caps. To 5.00 ml of an alkaline solution of known pH and composition was added 0.100 ml of peptide solution to give a final substrate concentration of 1–2 mM. The pH values of representative mixtures were rechecked after the addition of peptide. Incubations were conducted in separate tubes for each time interval. At selected intervals, 500-μl aliquots were removed from the reaction vessels and were diluted with an equal volume of 0.2 N citrate (pH 2.2). In those cases where highly buffered solutions were used, HCl had to be added to lower the pH sufficiently. Final analyses were conducted on a Beckman Model 120C automatic amino acid analyzer (Moore *et al.*, 1958). Pseudo-first-order hydrolysis constants were determined by least-squares analyses of plots of the log of the substrate concentration (log *A*) *vs.* time. In certain cases, the log of the initial peptide concentration minus the amount of substrate hydrolyzed (log $A_0 - x$) was plotted *vs.* time.

pH Measurements. pH determinations were made with a Leeds and Northrup expanded scale pH meter equipped with an A. H. Thomas combination electrode (No. 4858-L60, pH range 0-14). Above pH 12.0, the meter was standardized against a saturated $Ca(OH)_2$ solution which has a pH of 12.52 at 23° and a temperature coefficient ($\delta pH/\delta T$) of −0.033 pH unit per degree over the range 20–35°.

Measurement of Optical Rotation. Optical rotations were determined on a Cary Model 60 spectropolarimeter equipped with a thermostatically controlled cuvet holder.

TABLE I: The Influence of Various α Substituents on the Rates of Alkaline Hydrolysis of Dipeptides.[a]

Substrate	Hydrolysis Constants, $k \times 10^4$ hr^{-1}	Substituents[b] R′	R
Glycylglycine	28.0	H	H
Glycyl-L-alanine	8.3	H	CH_3
L-Alanylglycine	9.7	CH_3	H
Glycyl-L-leucine	2.1	H	$(CH_3)_2CHCH_2$
L-Leucylglycine	2.0	$(CH_3)_2CHCH_2$	H
Glycyl-L-valine	2.8	H	$(CH_3)_2CH$
Glycyl-D,L-α-aminobutyric acid	5.5	H	CH_3CH_2
Glycyl-L-serine	29.0	H	$HOCH_2$
L-Serylglycine	7.6	$HOCH_2$	H
Glycyl-L-threonine	9.2	H	$CH_3(OH)CH$
L-Alanyl-L-serine	11.8	CH_3	$HOCH_2$
L-Seryl-L-alanine[c]	1.4	$HOCH_2$	CH_3
L-Leucyl-L-serine	3.8	$(CH_3)_2CHCH_2$	$HOCH_2$
L-Seryl-L-leucine[c]	0.6	$HOCH_2$	$(CH_3)_2CHCH_2$

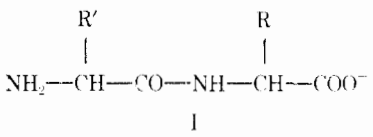
[a] Incubations performed at 30° in 0.03 M $KB(OH)_4$ (pH (12.6). [b] R and R′ refer to substituents in $NH_2CH(R')$-$CONHCH(R)COO^-$. [c] Corrected for epimerization according to the relationship: $k_{hyd} = k_{obsd} - k_e$.

Results

The Kinetics of Alkaline Hydrolysis of Dipeptides. Table I summarizes the influence of various α substituents on the pseudo-first-order hydrolysis constants of several alkyl and hydroxyalkyl dipeptides. Comparison of these hydrolysis constants reveals that the stability of the alkyl-substituted dipeptides is a function of the size of the substitu-

[2] Abbreviation used is: *t*-BOC, N^{α}-*tert*-butyloxycarbonyl.

ents (R and R′ in compound I) attached to the α-carbon



I

atoms. Thus, at pH 12.6 and 30°, glycyl-L-leucine ($k \times 10^4$ = 2.1 hr^{-1}) is approximately 13 times more resistant to alkaline hydrolysis than diglycine ($k \times 10^4$ = 28.0 hr^{-1}). When the various R groups are listed according to their stabilizing effects, the following order is obtained: $(CH_3)_2CHCH_2 \geq (CH_3)_2CH > CH_3CH_2 > CH_3 > H$.

Further comparison of the rate constants for the aliphatic dipeptides demonstrates that the order of stabilization by alkyl R groups remains unchanged regardless of whether the substituents occupy the R or R′ positions. Moreover, the rates are quantitatively similar regardless of the α carbon occupied. The latter point is strikingly illustrated by comparing the values for the hydrolysis constants obtained with the methyl-substituted dipeptides, glycyl-L-alanine ($k \times 10^4$ = 8.3 hr^{-1}) and L-alanylglycine ($k \times 10^4$ = 9.7 hr^{-1}); and also with the isobutyl-substituted compounds, glycyl-L-leucine ($k \times 10^4$ = 2.1 hr^{-1}) and L-leucylglycine ($k \times 10^4$ = 2.0 hr^{-1}).

Examination of the hydrolytic constants of dipeptides containing a hydroxyalkyl group (R(HO)CH–, where R = H or CH_3) reveals that the latter type of substituent can exert either a stabilizing or a labilizing effect on the peptide bond, depending on whether it is present as R or R′ (see compound I). For example, the rate of hydrolysis of L-seryl-glycine ($k \times 10^4$ = 7.6 hr^{-1}) is about four times slower than that of glycylglycine ($k \times 10^4$ = 28.0 hr^{-1}) and approximately as fast as that of L-alanylglycine ($k \times 10^4$ = 9.7 hr^{-1}). Glycyl-L-serine ($k \times 10^4$ = 29.0 hr^{-1}), on the other hand, is hydrolyzed as fast as glycylglycine even though the R substituent ($HOCH_2$–) is bulkier than that found in glycyl-L-alanine ($k \times 10^4$ = 8.3 hr^{-1}). Glycyl-D,L-α-aminobutyrate, the methyl analog of glycyl-L-serine, is hydrolyzed at only one-fifth the rate ($k \times 10^4$ = 5.5 hr^{-1}) of the latter. Similarly, glycyl-L-threonine ($k \times 10^4$ = 9.2 hr^{-1}) is hydrolyzed about three times more rapidly than its methyl analog, glycyl-L-valine ($k \times 10^4$ = 2.8 hr^{-1}).

Additionally significant is the finding that the occurrence of bulky alkyl substituents in the R′ position of serine dipeptides creates an impediment to hydrolysis which overrides the catalytic influence of the hydroxymethyl group. Consequently, L-alanyl-L-serine and L-leucyl-L-serine are 2–7 times more resistant to hydrolysis than glycyl-L-serine.

The Alkali-Catalyzed Epimerization of Hydroxymethyl-Containing Dipeptides. During the process of investigating the alkaline hydrolysis of hydroxymethyl-containing dipeptides, it was observed that L-seryl-L-alanine and L-seryl-L-leucine, in addition to undergoing simple hydrolysis, selectively epimerized at the R′ position to form D,L diastereomers. The rate of epimerization at 30° and pH 12.6 was determined to be the same for both of the aforementioned L,L dipeptides ($k_e \times 10^4$ = 1.3 hr^{-1}).

In order to establish the site of epimerization, L-seryl-L-leucine[3] was incubated for 24 hr at 90° and pH 12.6 in 0.03 M $KB(OH)_4$. The resultant diastereomers plus their hydrolysis products were then isolated by ion exchange chromatography (for details, see the Materials and Methods section). After recrystallization, both diastereomers were found to contain equimolar amounts of serine and leucine, and both gave the same elemental analysis. The serine recovered from the alkaline incubation mixture gave a specific rotation ($[\alpha]^{23}_{589}$) in water of −4.6° which was significantly different than the −7.8° reported by Greenstein and Winitz (1961) for the L-amino acid. In the case of leucine, the $[\alpha]_{589}$ of −10.7° (Greenstein and Winitz, 1961) remained essentially unchanged. This evidence was taken to indicate that serine rather than leucine had undergone racemization *in alkali* to form D-seryl-L-leucine. Furthermore, the formation of L-seryl-D-leucine was not expected because of the lack of influence of the carboxylate group on the ionization of the α hydrogen to bring about α-carbanion formation (Neuberger, 1948; Desnuelle, 1953).

The reversibility of the alkali-catalyzed epimerization of seryl-containing dipeptides is demonstrated in Figure 1 for L-seryl-L-leucine (A) and D-seryl-L-leucine (B). An approximation of the *initial* pseudo-first-order rate constants for substrate decay, epimerization, and hydrolysis indicates *little difference, if any, in the various reaction velocities* associated with the L,L and the D,L diastereomers (Table II). The differences in stoichiometry between the hydrolyzed serine and leucine in Figure 1 is due to the partial decomposition of serine in alkali (Neuberger, 1948).

The Mechanism of Peptide Bond Hydrolysis. In a previous communication, it was postulated that certain buffers (*e.g.*, borate, phosphate, etc.) may act as polyfunctional catalysts in the selective cleavage of peptidyl serine and threonine bonds in proteins (Sine and Hass, 1969). In order to test this hypothesis, glycyl-L-serine was incubated under alkaline conditions and constant ionic strength with increasing concentrations of either borate or phosphate. As shown in Figure 2, each of the above substances had virtually no influence on the k_{obsd}, indicating that the aforementioned hypothesis is invalid. In the presence of phosphate buffer, slightly greater variations in k_{obsd} were noted, but these were ascribed to fluctuations in pH and ionic effects caused by the concentration-dependent pK_3 of orthophosphate.

Having determined that the presence of buffer plays no role in the alkaline cleavage of peptidyl-serine bonds, we further investigated the hydrolysis of dipeptides to determine whether spontaneous catalysis (*i.e.*, water catalysis) was involved.

Under alkaline conditions, it can be shown that the pseudo-first-order hydrolysis constant for acyl compounds obeys the following expression (Bruice and Benkovic, 1966)

$$k_{obsd} = k_0 + k_{OH}K_w/a_H = k_0 + k_{OH}a_{OH}$$

where k_0 equals the rate constant for solvent catalysis and k_{OH} is the rate constant for specific base catalysis. K_w is the autoprotolysis constant for water. If there is no water catalysis, it follows that $\log k_{obsd} = \log k_{OH}K_w + pH$, so that a plot of $\log k_{obsd}$ *vs.* pH should be linear with a slope of +1.0.

Figure 3 shows $\log k_{obsd}$ for the alkaline hydrolysis of glycyl-L-serine and diglycine as a function of pH at 25° and constant ionic strength. As illustrated, the slopes of both substrates essentially equal unity, indicating that, under the conditions cited, water plays no role in the catalysis. If the data in Figure 3 are replotted as k_{obsd} *vs.* K_w/a_H (not shown), the lines for both substrates pass through the origin of the graph, leaving little doubt that k_0 is indeed negligi-

[3] L-Seryl-L-leucine was chosen for investigation because of its relative resistance to alkaline hydrolysis. See Table I.

TABLE II: Pseudo-First-Order Rate Constants for the Decay, Epimerization, and Hydrolysis of L-Seryl-L-leucine and D-Seryl-L-leucine.[a]

Rate	$k \times 10^2$ hr^{-1}	
	L-Ser-L-Leu	D-Ser-L-Leu
Decay[b]	4.2	4.2
Epimerization	3.3	3.4
Hydrolysis[c]	0.55	0.53

[a] Incubations performed at 90° in 0.03 M $KB(OH)_4$ (pH 12.6). [b] Decay refers to epimerization plus hydrolysis. [c] Based on leucine release (see text).

ble. The slopes of the latter plots give 3.6×10^{-2} and 3.3×10^{-2} M^{-1} hr^{-1} which are the second-order rate constants for the specific base-catalyzed hydrolysis of glycyl-L-serine and diglycine, respectively.

The Kinetics of Tripeptide Racemization and Hydrolysis in Alkali. In an effort to determine whether certain principles found for dipeptides are applicable to larger peptides, the rates of alkali-catalyzed decay (*i.e.*, hydrolysis plus epimerization) of gylcyl-L-seryl-L-alanine and L-alanyl-L-serylglycine have been studied.

Figure 4 illustrates the kinetics obtained when glycyl-L-seryl-L-alanine is exposed to 0.03 M $KB(OH)_4$ at pH 12.6 and 30°. Analysis of the rates of product formation shows that the glycyl-serine bond ($k \times 10^3 = 3.8$ hr^{-1}) is cleaved at approximately four times the rate of the seryl-alanine bond ($k \times 10^3 = 1.1$ hr^{-1}) as might be predicted from dipeptide studies. Comparison of the above k values with those for dipeptides in Table I, however, reveals that the hydrolytic rates of both bonds are increased in tripeptides, but in the case of the seryl-alanine bond, the increase is an unexpected 7.9-fold as compared with a 1.3-fold for the glycyl-serine bond.

Figure 4A shows no evidence of epimerization except for the *apparent* slow disappearance of tripeptide and the relatively rapid appearance of glycine compared to that of L-seryl-L-alanine. The difference in the kinetic curves of the latter two products has been assumed to indicate the formation of D-seryl-L-alanine (dashed curve). If our assumption is correct, D-seryl-L-alanine must arise from the glycyl-D-seryl-L-alanine epimer produced during the incubation process, since the rate of formation of the former compound increases rather than decreases with time. Thus, it has been reasoned that the decay curve (solid triangles) represents glycyl-D,L-seryl-L-alanine and D-seryl-L-alanine which are eluted coincidentally on the amino acid analyzer. Figure 4B shows that all materials can be accounted for when appropriate corrections are made based on the above hypothesis. Figure 4B also manifests the concomitant rates of tripeptide degradation and dipeptide formation ($k \times 10^3 = 5.5$ hr^{-1}).

Figure 5 illustrates the kinetics obtained when L-alanyl-L-serylglycine is exposed to the alkaline conditions cited in Figure 4. Analysis of the rates of product formation shows that the alanyl-serine bond ($k \times 10^3 = 2.9$ hr^{-1}) is hydrolyzed at a slightly greater rate than the seryl-glycine bond ($k \times 10^3 = 2.2$ hr^{-1}). This finding is also in accord with predictions based on the results obtained with dipeptides (see Table I). As with glycyl-L-seryl-L-alanine, the hydrolysis constants of both peptide bonds are increased over those obtained with dipeptides, but in this case, the increases are approximately equivalent, being 2.5-fold for alanyl-serine and 2.8-fold for seryl-glycine.

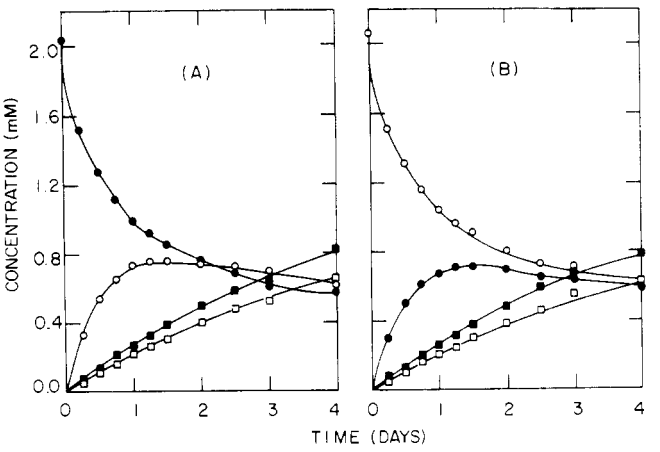


FIGURE 1: Comparison of the kinetics of epimerization and hydrolysis of L-seryl-L-leucine (A) and D-seryl-L-leucine (B). Substrates (2 mM) were incubated at 90° in 0.03 M potassium borate at pH 12.6 according to procedures described under Materials and Methods. ●, ○, ■, and □ respectively represent L-seryl-L-leucine, L-seryl-D-leucine, L-leucine, and D,L-serine.

Unlike Figure 4A, Figure 5A provides obvious evidence for epimerization through the kinetic curves established for the formation of L-alanyl-D-serine and L-alanyl-D-serylglycine. The constant for the initial rate of epimerization of L-alanyl-L-serylglycine at pH 1.26 and 30° is 2.1×10^{-2} hr^{-1} which is about 160-fold greater than the 1.3×10^{-4} hr^{-1} obtained with the dipeptides L-seryl-L-alanine and L-seryl-L-leucine under equivalent conditions.

Figure 5B represents an accounting of all materials after alkaline treatment; it also manifests the rate of tripeptide degradation to dipeptides ($k \times 10^3 = 5.2$ hr^{-1}).

Discussion

The studies described here and elsewhere (Levene *et al.*, 1932; Jarboe *et al.*, 1971) have shown that the alkaline stability of the amide bond in alkyl-substituted peptides is a function of the size of the R substituents. When the various R groups are listed according to their stabilizing effects, the order cited in the text is obtained. This order corresponds exactly to that established for alkyl groups when they are

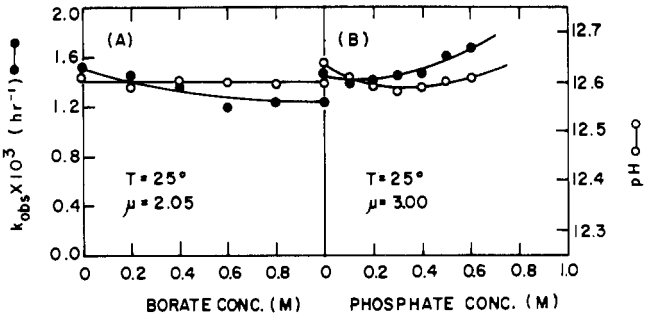


FIGURE 2: The effect of borate (A) and phosphate (B) concentration on the rate of alkaline hydrolysis of glycyl-L-serine. The left-hand ordinate represents the observed pseudo-first-order rate constant (●), while the right-hand ordinate indicates the pH (○). The substrate (2 mM) was incubated at 25.00 ± 0.001°. The pH was obtained with KOH and was measured before and after each incubation. Addition of KCl was used to maintain an ionic strength of 2.05 and 3.00 M in (A) and (B), respectively. In (B) a pK_3 of 12.67 for HPO_4^{2-} was assumed. Incubation procedures were conducted as described in the Materials and Methods section.

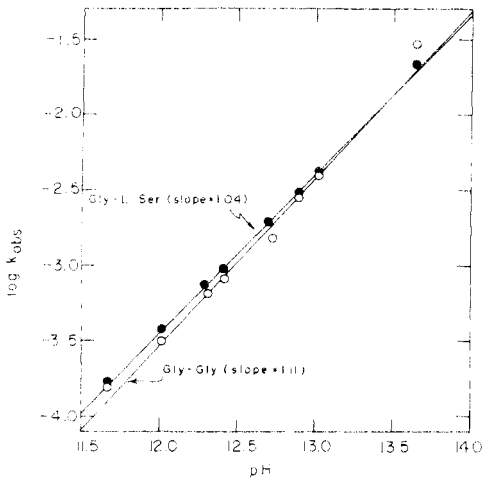


FIGURE 3: Plot of the log of the observed first-order hydrolysis constant *vs.* alkaline pH values for glycyl-L-serine (●) and glycylglycine (○). The dipeptides (2.0 mM) were incubated in KOH-KCl at 25° and a constant ionic strength (μ) of 0.1 M. At pH 13.65, μ = KOH concentration ≃0.45 M. Incubation procedures were conducted as described in the Materials and Methods section.

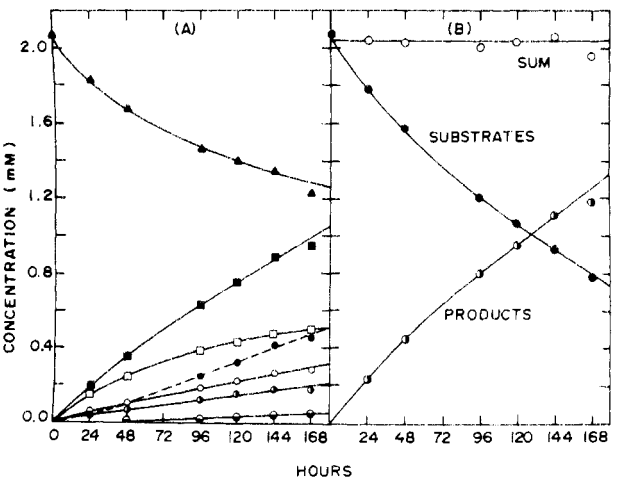
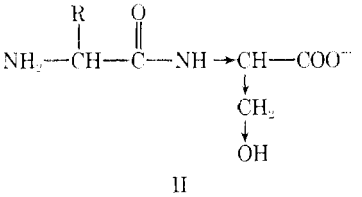


FIGURE 4: The influence of dilute alkali on the kinetics of hydrolysis of glycyl-L-seryl-L-alanine. The substrate (2.0 mM) was incubated in 30 mM potassium borate at pH 12.6 and 30° according to procedures described under Materials and Methods. (A) Experimentally determined curves for: (1) D,L-serine, ⊖; (2) glycyl-D,L-serine, ◑; (3) L-alanine, ○; (4) L-seryl-L-alanine, □; (5) glycine, ■; (6) glycyl-D,L-seryl-L-alanine plus D-seryl-L-alanine, ▲; and (7) D-seryl-L-alanine ● (calculated as 5 minus 4). (B) Calculated curves for: unhydrolyzed tripeptides, ● (6 minus 7 from Figure A); hydrolyzed tripeptides, ◑ (5 plus 3 minus 1 from Figure A); and the sum of hydrolyzed and unhydrolyzed tripeptides, ○.

listed according to their electron donating or withdrawing capacities (Huheey, 1971). It would seem reasonable, therefore, to postulate that a positive inductive effect might account for the hydrolytic resistance of the alkyl-substituted peptides. On the other hand, the second ionization constants (pK_2) of the alkyl amino acids are not affected significantly by the different sizes of the R substituents, indicating, contrary to expectancy, that induction plays no role in the dissociation properties of the RNH_3^+ group (Cohn and Edsall, 1948). Thus, by analogy with the latter phenomenon, the principal function of the alkyl R group in stabilizing peptide bonds is more than likely steric rather than inductive.

Of considerable significance is the finding that homologous alkylglycine and alkylserine compounds have essentially equivalent hydrolysis constants (Table I). Thus it appears that despite its bulkiness, the occurrence of serine in the COOH-terminal position of dipeptides has about the same influence as glycine on the stability of the amide bond in alkali. Since neither borate nor phosphate has any catalytic effect on peptide bond hydrolysis and, therefore, cannot be involved in the stabilization of a hydroxyoxazolidine intermediate, serine's hydroxyl group undoubtedly does not participate in a mechanism which requires an N → O shift as suggested earlier by Sine and Hass (1969). Instead, the OH group might play a role which is based solely on its electron withdrawing capacity. This capacity (or negative inductive effect) is envisioned as restricting the resonance contribution of the peptide nitrogen, thereby enhancing nucleophilic attack at the peptide carbonyl by OH^- (see II).



II

Support for the inductive capacity of the hydroxymethyl group of serine is obtained by comparing the hydrolysis constants for glycyl-L-serine ($k \times 10^4 = 29.0\ hr^{-1}$), glycyl-L-threonine ($k \times 10^4 = 9.2\ hr^{-1}$), and glycyl-L-valine ($k \times 10^4 = 2.8\ hr^{-1}$). When threonine is substituted for serine in glycyl dipeptides, cleavage is dampened by about threefold. That the resultant effect is both inductive and steric becomes obviously apparent when it is realized that the rate of cleavage of the methyl analog of glycyl-L-threonine (glycyl-L-valine) is further reduced by an inordinate 3.3-fold despite the slight difference in volume between CH_3 and OH. Hence, the catalytic properties of the hydroxymethyl group still remain operative in threonine, but are partially offset through the insertion of CH_3 into the side chain.

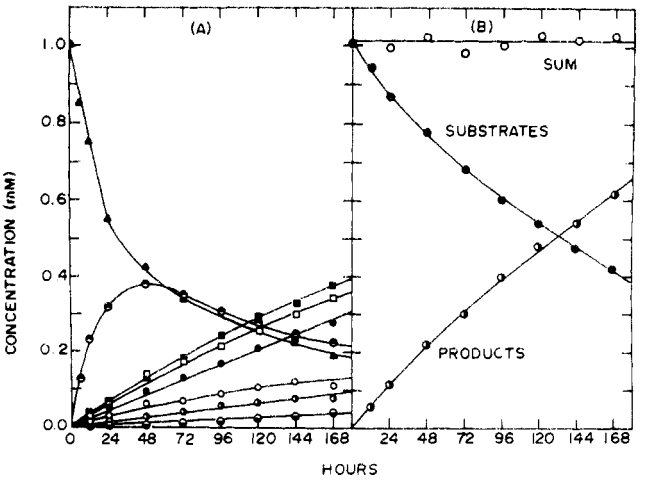
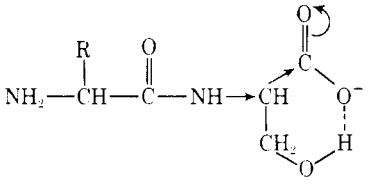


FIGURE 5: The influence of dilute alkali on the kinetics of hydrolysis and epimerization of L-alanyl-L-serylglycine. The substrate (1.0 mM) was incubated under the conditions given in Figure 4. (A) Experimentally determined curves for: (1) D,L-serine, ⊖; (2) L-alanyl-D-serine, ◑; (3) L-alanyl-L-serine, ○; (4) glycine, ●; (5) D,L-serylglycine, □; (6) L-alanine, ■; (7) L-alanyl-D-serylglycine, ⊖; and (8) L-alanyl-L-serylglycine, ▲. (B) Calculated curves for: unhydrolyzed tripeptides, ● (7 plus 8 from Figure A); hydrolyzed tripeptides, ◑ (6 plus 4 minus 1 from Figure A); and the sum of hydrolyzed and unhydrolyzed tripeptides, ○.

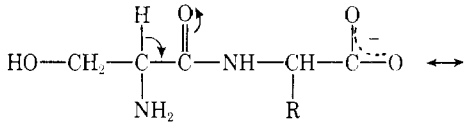
An alternate mechanism provides for H-bonding between the serine's OH and the dipeptide's COO^- (see III). In this



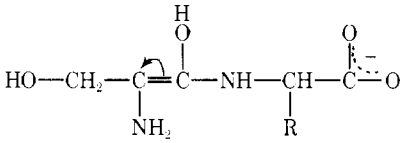
III

case, the COO^- is rendered more ester like, thus creating a short range inductive effect which could facilitate peptide bond scission in alkali. This mechanism should also promote racemization of the serine moiety, creating L-aminoacyl-D-serine compounds; a phenomenon for which we find no evidence.

Serine in the NH_2-terminal position of dipeptides imparts an unusual degree of hydrolytic resistance to the amide bond (Table I) and also promotes formation of the diastereoisomer of the parent compound (Figure 1). Thus under alkaline conditions, a pair of electrons is freed to participate in the concomitant stabilization of the peptide bond and the racemization of the parent compound. The lability of the α hydrogen is promoted by the combined inductomeric effect resulting from the serine OH and the tautomeric effect involving the amide carbonyl. These effects are depicted in the tautomeric forms IV and V.

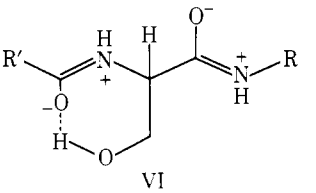


IV



V

It is noteworthy that, under equivalent conditions, the initial rate of racemization of the internally located seryl residues in tripeptides is 160-fold greater than its counterpart in dipeptides. The highly acidic nature of X-seryl-Y is probably best explained by the diallylic nature of the serine's α carbon. Furthermore, it seems reasonable to postulate that one of the allylic groups is stabilized through H-bonding between serine's OH and the oxygen of an adjacent carbonyl (see VI) (Pullman and Pullman, 1974).



VI

Although it has been demonstrated that under mildly alkaline conditions large proteins invariably cleave at peptidyl-glycine, -serine, and -threonine bonds (Sine and Hass, 1969; Hass *et al.*, 1968), the principles stated here for di- and tripeptides remain to be established for intact proteins. This might readily be accomplished by identifying the resultant oligopeptides obtained after exposing proteins of known amino acid sequences to dilute alkali.

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