might be due, in part, to their ability to reduce the thickness of the reaction barriers, thereby promoting nuclear tunneling by hydrogen. However, the probability of tunneling is at a maximum only for a symmetric transition state,[43] and we indeed observe that the enzymatic reaction, in sharp contrast to the nonenzymatic reaction, has a more symmetric transition state.

Coenzyme Variation–Enzymatic PCA Reaction Rates. We have found that the reduction of the imino acid proceeds 10 times faster with E–NADH than with enzyme-reduced 3-acetylpyridine adenine dinucleotide (E–3-APADH) and 8 times[44] faster with NaDH than with 3-APADH. Since the substituent changes in the coenzymes are made close to the reaction site, we have abandoned the use of β values calculated from these reactivity ratios to describe the transition-state structures.[45] Instead, we have assumed that the relative changes in $\Delta G^{\ddagger}$ due to the nonelectronic effects of the amide and acetyl groups in the coenzymes are the same in the nonenzymatic and enzymatic reactions. In view of the transition state for the enzymatic reaction being less product-like than the nonenzymatic reaction, it is anomalous that the rates for the enzymatic reaction are more sensitive to coenzyme variation. We ascribe this anomaly to the unusually low reactivity of E–3-APADH and suggest[47] that 3-APADH, having no amide group, is unable to bind to the enzyme in a favorable fashion to derive entropic advantage of the subsequent step (which involves binding to PCA and reacting). There is in fact spectroscopic evidence[48] showing that there is interaction between the dihydropyridine ring and the enzyme in E–NADH but not in E–3-APADH. Furthermore, we have found that the enhanced reactivity of E–NADH over NADH itself in the PCA reaction is entirely due to a more favorable entropy of activation with the former reductant.[49]

Comparison to the Glutamate Reaction. The largest deuterium isotope effect observed for the glutamate dehydrogenase catalyzed reversible oxidative deamination of L-glutamate occurs in the initial burst produced by the enzyme–$NADP^+$–glutamate complex under single turnover conditions.[50] An isotope effect of 1.5 to 1.8 was measured[51] on this initial burst of the reaction with L-glutamate-*2-d*. If we assume that the isotope effect of 4.1 measured for the oxidation of proline and proline-*2-d* represents the intrinsic isotope effect for the glutamate oxidation then the hydride-transfer process must limit the initial burst rate by 16–26%. Clearly, the remaining contribution to rate-limitation is made by the forward commitment, involving partition ratios for steps up to the color-producing one[52] (λ_{max} 332 nm[51]).

Acknowledgment. This work was supported in part by Grant PCM-8203880 from the National Science Foundation, Grant GM-15188 from the General Medicine Institute of the National Institutes of Health, and the Veterans Administration. We thank Dr. S. Pazhanisamy for helpful discussions.

Registry No. [4-^{2}H]**1**, 17750-27-5; [4,4-^{2}H]**2**, 60172-94-3; [4,4-^{2}H]**3**, 96555-70-3; [4,4-^{2}H]**4**, 60764-22-9; PCA, 2139-03-9; deuterium, 7782-39-0; glutamate dehydrogenase, 9029-12-3.

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(44) The effect of the 3-acetyl group in relation to the 3-amide group in the reductant on the nonenzymatic reaction rates is independent of the *N*-1 substituent. When the 3-substituent in 1-(carbamoylmethyl)-3-carbamoyl-1,4-dihydropyridine is changed from amide to acetyl the rate falls off by 8.9-fold,[5] in good agreement with the 8.3-fold we observe with NADH and 3-APADH.
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Specific Cleavage of Peptides Containing an Aspartic Acid (β-Hydroxamic Acid) Residue

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Abstract: Peptides containing the aspartyl β-hydroxamic acid residue are cleaved specifically at the carboxyl side of this residue at pH values ≥6. The cleavage occurs by attack of both the hydroxamic acid nitrogen and the hydroxamic acid oxygen to yield tetrahedral intermediates that, in the rate-limiting step, break down with cleavage of the peptide chain. In a competing reaction, the peptide nitrogen on the carboxyl side attacks the hydroxamate carbonyl to expel hydroxylamine and give an imide intermediate **28** (Scheme I). The cleavage yield reflects the relative efficiency of these two pathways. The extent of cleavage is dramatically increased in the presence of 1 M added hydroxylamine. The extent of cleavage is also increased significantly by phosphate buffers, but not by PIPES or imidazole buffers, in the absence of added hydroxylamine. The role of hydroxylamine in some cases may be to intercept the imide **28** or isoimide **33** (eq 8); but in at least two cases evidence is presented that hydroxylamine, like phosphate, may be acting as a general acid–base catalyst that selectively catalyzes the breakdown of tetrahedral intermediates **23** or **24** (Scheme I) leading to chain cleavage. Peptides containing glutamyl (γ-hydroxamic acid) residues are also cleaved, but at rates that are 20–40 times slower than those of the analogous aspartyl peptides. The results of this work completely rationalize in a mechanistic sense the hydroxylamine cleavage of Bornstein and Balian[6] and suggest a general method for the cleavage of peptides at aspartic acid residues.

About 1960 it was first recognized that peptides and proteins could be cleaved specifically with alkaline solutions of hydroxylamine under relatively mild conditions.[2] In 1969 Butler[3] and Bornstein[4] established that fragments of the α1 chain of rat

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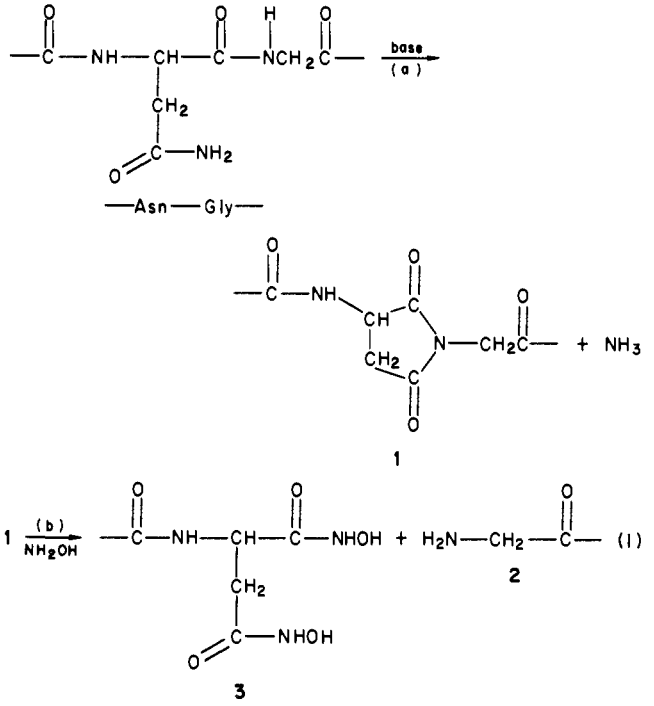
collagen obtained by CNBr cleavage could be cleaved with high specificity at the asparaginyl–glycine (Asn–Gly) bonds by incubating the peptides with 1–2 M hydroxylamine under mildly alkaline conditions. Similarly, the single Asn–Gly bond in ribonuclease can be cleaved by alkaline hydroxylamine.[5] Bornstein and Balian[6] have summarized the detailed conditions for this cleavage, which has now become standard in peptide-sequencing methodology.[7]

A mechanism for this cleavage not only shows why it is selective for Asn–Gly bonds but also suggests, as we shall detail below, an extension to a general cleavage of peptides at Asp residues. This cleavage takes place in essentially two major steps. In step (a) an *imide intermediate* **1** is formed, and in step (b) this intermediate is cleaved, giving a peptide with amino-terminal glycine **2** and a peptide containing the labile Asp residue as a C-terminal dihydroxamic acid **3**.



It is well known that imide formation is particularly facile at Asn–Gly sequences and at Asp–Gly sequences in which the Asp side-chain carboxyl group is esterified; this phenomenon is particularly annoying in the synthesis of Asx–Gly bonds.[8] It is the

Table I. Specific Cleavage at 60 °C of Peptides Containing an Aspartic Acid(β-Hydroxamic Acid) Residue[a]

compd no.	peptide (pH)		$10^4 k_{obs}$,[b] s^{-1}	max % cleavage[c]
6	*N*-Ac-Asp-Ala	(9.5)	3.3	65
	NHOH	(6.8)	0.51	57
7	*N*-Ac-Asp-NHOH	(9.7)	1.9	46
	Ala	(6.9)	0.64	44
8	*N*-Ac-Asp-Ala	(9.5)	0.11	8
	NH—OCH_3	(6.8)	0.069	30
9	*N*-Ac-Asp-Ala	(9.5)		0
	$N(CH_3)OH$	(6.8)	0.036	20
10	*N*-Ac-Asp-D-Ala	(9.5)	2.9	77
	NHOH	(6.9)	0.78	66
11	*N*-Ac-Asp-Phe	(9.9)	0.97	71
	NHOH	(6.9)	0.23	67
12	*N*-Ac-Asp-Ala-Gly	(9.5)	2.6	20
	NHOH	(6.7)	0.56	21
13	*N*-Ac-D,L-Asp-Val-Ala	(9.4)	0.97	60
	NHOH	(6.8)	0.12	38
14	*N*-Ac-Asp-Gly	(9.5)	41.	16
	NHOH	(6.8)	2.0	53
31	*N*-Ac-Asp-Pro	(9.4)	2.4	87
	NHOH	(6.8)	0.37	92

[a] Buffers used: 0.05 M $NaHCO_3$ at pH 9–10; 0.1 M NaH_2PO_4 at pH 6.8. The measured pH (in parentheses) is that determined at 60 °C. [b] Pseudo-first-order rate constant for the observed reaction. Rate constant for cleavage equals this rate constant times the fraction of cleavage observed.[16] Reproducibility of rates was ±15%. [c] Reproducibility of cleavage yields was ±5%.

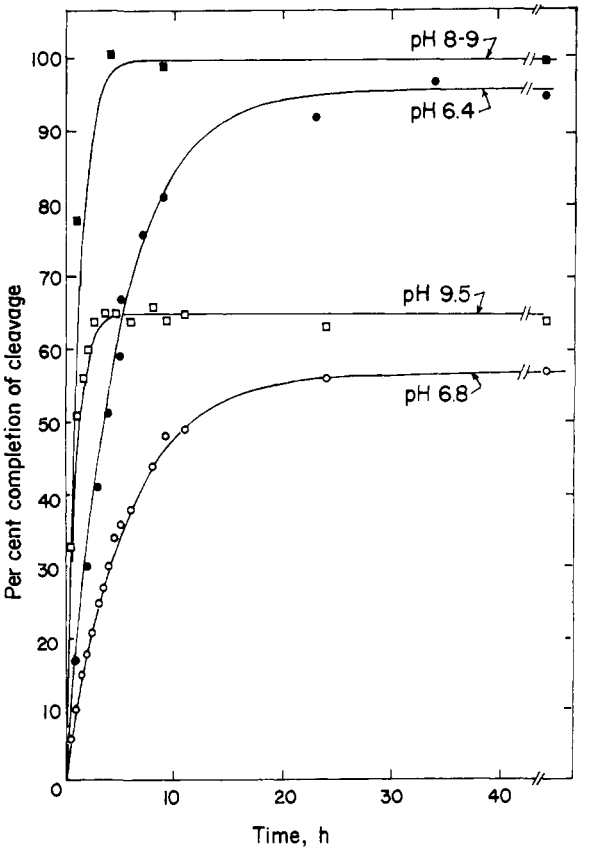


Figure 1. Cleavage of *N*-Ac-Asp(β-hydroxamic acid)-Ala **6** at various pH values in the presence and absence of hydroxylamine. Open symbols: no added hydroxylamine; the buffers used are given in Table I. Filled symbols: 1 M hydroxylamine added and used as buffer. Circles: pH 6–7. Squares: pH 8–10. The curves are drawn from the rate constants given in Tables I and III.

(1) (a) Purdue University. (b) Johns Hopkins University. (c) This work was taken in part from a thesis of James K. Blodgett submitted to the Graduate School of Purdue University in partial fulfillment of requirements for the M.S. degree.

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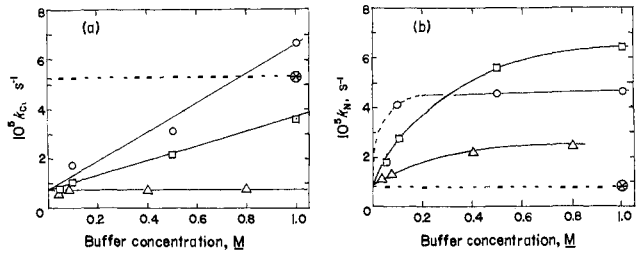
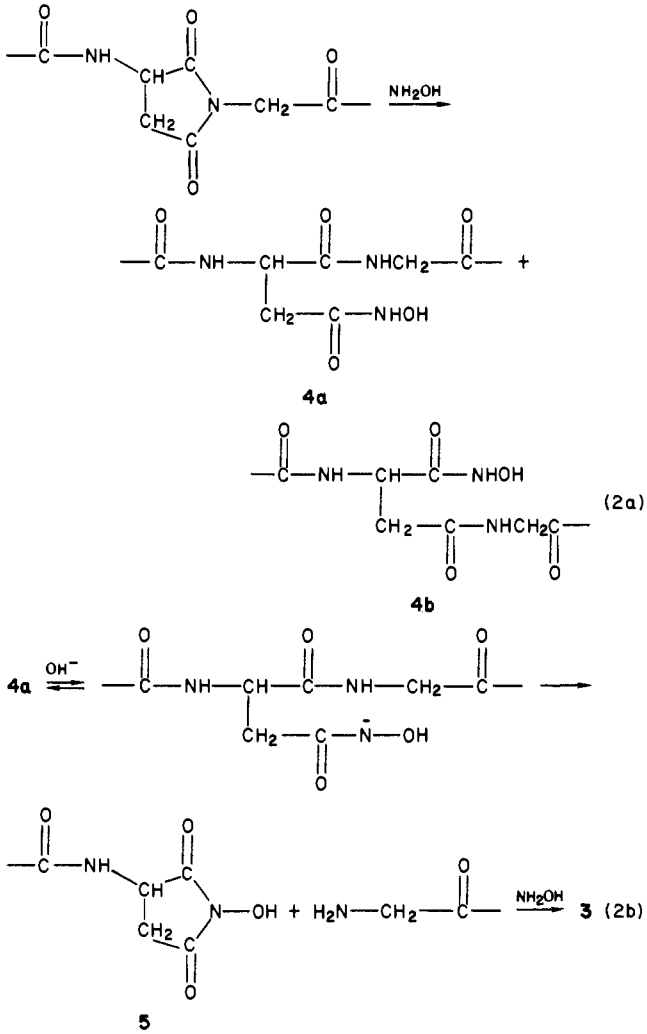


Figure 2. Rates of reaction of *N*-Ac-Asp(β-hydroxamic acid)-Ala-Gly (**12**) at pH 6.3 as a function of total buffer concentration: (○) phosphate buffer; (□) imidazole buffer ; (△) PIPES buffer. The asterisks are the points for 1 M hydroxylamine. (a) Cleavage rate constants, k_C, calculated by multiplying k_{obsd} (Table IV) times the fraction cleavage observed at infinite time. (b) Rate constant for competing pathways, k_N, calculated as the difference between k_{obsd} and k_C. The lines are smooth curves connecting the points, and the dashed line for phosphate is drawn under the assumption that all reactions have the same rate at zero buffer.

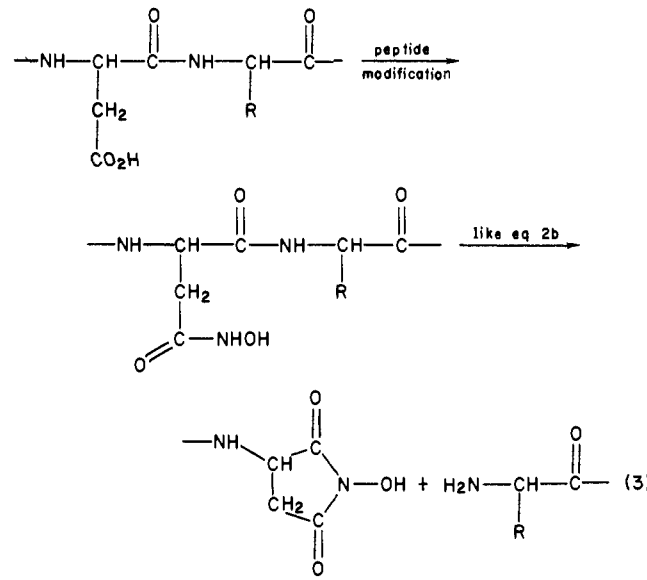
unique susceptibility of Asn–Gly bonds toward imide formation that accounts for the selectivity of the hydroxylamine cleavage. The evidence for imide formation in such reactions has been detailed in several instances.[6,8g]

It is in step (b) that the actual cleavage occurs; yet the details of this step are not clear. An understanding of this step is crucial not only to understanding how the actual peptide cleavage occurs but also, as we shall detail below, to the development of a general peptide cleavage at Asp residues. The following is a rational mechanism for step (b) and is different from that presented by Bornstein and Balian.[6]



In this mechanism the side-chain hydroxamic acid function displaces the glycine amino group. (A similar mechanism would be operative for the isomeric peptide **4b**. Furthermore, attack of the hydroxamate oxygen in **4a** or **4b** or attack of the carbonyl oxygen of the hydroxamate group are other mechanisms that would result in the same cleavage.)

An interesting point about this cleavage mechanism is that eq 2b might be general for the peptide bond at *any* Asp residue, regardless of sequence. According to this hypothesis, the Asn–Gly cleavage is specific because of imide formation, and the role of the imide is to activate side-chain carbonyl groups of Asn residues in the peptide Asn–Gly linkages toward nucleophilic incorporation of hydroxylamine. However, if one could introduce a side-chain hydroxamic acid group into other aspartyl residues, the mechanism in eq 2b suggests that cleavage should be observed under alkaline conditions at these residues as well:

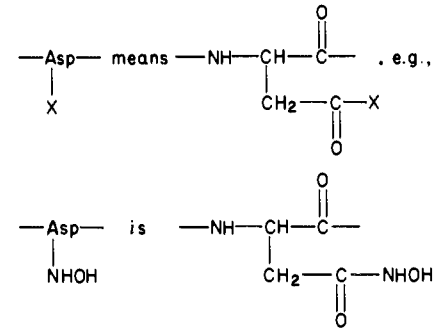


The substantial interest that has been expressed in the development of a peptide cleavage at aspartyl residues[9] suggests that the chemistry shown in eq 3 might be developed into a generally useful protocol of peptide sequencing.

Essential to the development of a specific cleavage at Asp by modification of Asp residues to Asp(β-hydroxamic acid) residues is the demonstration that the cleavage of peptides at such residues as shown in eq 2b and 3 is general. In this paper we report our investigations of the selective cleavage of peptides containing Asp(β-hydroxamic acid) residues, the possible role of these compounds in the Bornstein–Balian cleavage,[6] and their potential as intermediates in a general peptide cleavage at aspartic acid.

Results and Discussion

Notation. In order to simplify structures the following notation for Asp- and Glu-containing peptides will be used to indicate derivatization of the carboxyl groups.



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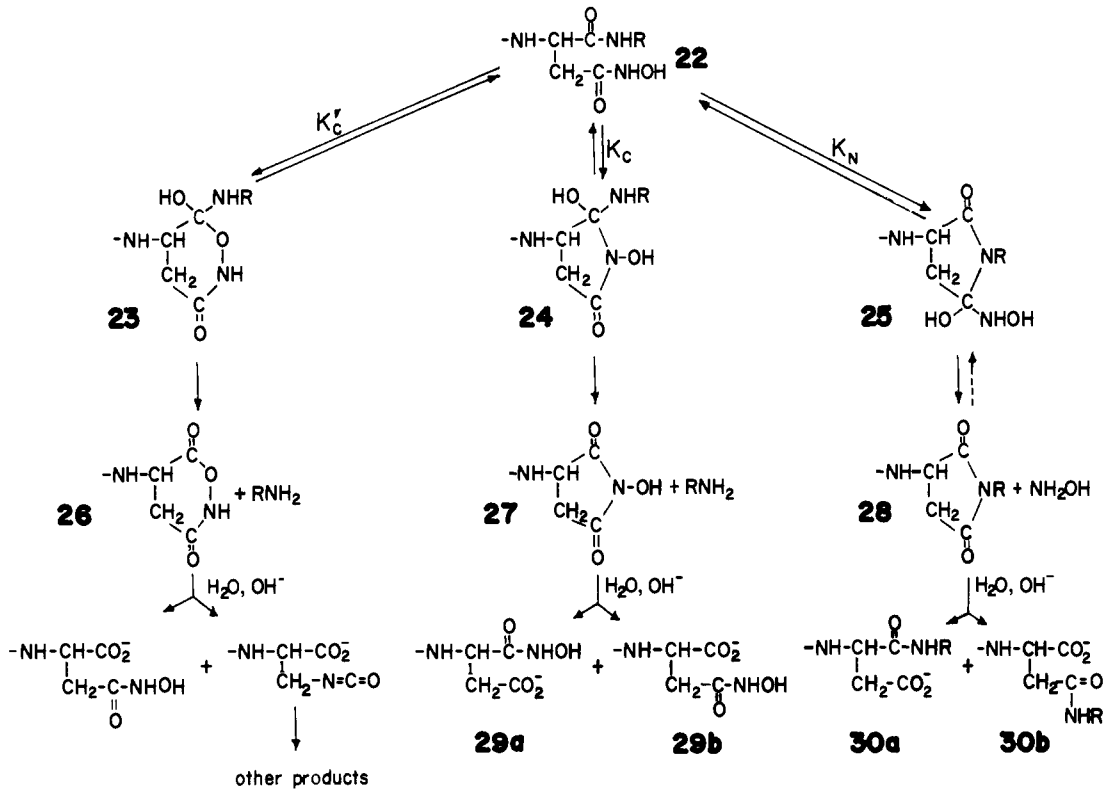
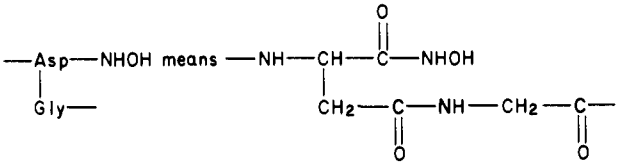
Scheme I



The corresponding β-linked peptides will be abbreviated in a similar way:



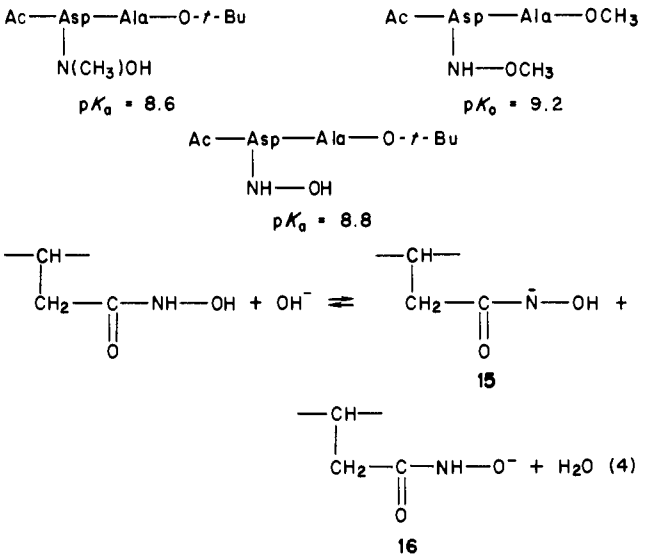
All amino acids are of the L configuration unless otherwise indicated.

Methodology. Peptides were synthesized by solution methods. For the analysis of peptide cleavage a solution that was 2–8 mM in starting peptide containing an internal standard was allowed to react under the appropriate conditions at 60 °C. An aliquot of this solution was subjected directly to amino acid analysis (ninhydrin detection) without hydrolysis, and the products were analyzed directly by integration of the appropriate peak. The color values were normalized to recently run standards. For certain peptides (**6**, **11**, **14**, **31**, **35–37**) it was verified that their cleavage reactions follow a pseudo-first-order rate law by plotting log ([products at $t = \infty$] − [products at t]) vs. time t. For most peptides, the rates of reaction were calculated from the half-lives for appearance of products; in obtaining data we focussed on an accurate determination of maximum percent cleavage rather than a precise rate since it is the former that will likely be most significant in practical applications resulting from this work.

Cleavage of Peptides Containing Aspartyl (β-Hydroxamic Acid) Residues. Liberation of free alanine by the cleavage of the peptide **6** (Table I) containing a side-chain hydroxamic acid group as a function of pH and time at 60 °C is typical and is shown in Figure 1. That the control peptide Ac-Asp-Ala shows no hydrolysis whatsoever under identical conditions at pH ≥5.7 demonstrates that the observed cleavage at these pH values is specific and attributable to the presence of the side-chain hydroxamate group. Similarly, a control asparagine peptide, Ac-Asn-Phe, gave only 2% cleavage to Phe after the corresponding hydroxamate derivative had reacted for 4 half-lives. As the pH is lowered liberation of Ala from the control Ac-Asp-Ala became progressively more rapid. This observation is probably attributable to the cleavage at Asp residues that is known to occur in dilute acid.[9c,d] These results indicate that the hydroxamate-specific cleavage can be studied at pH ≥6.

Data for the cleavage of hydroxamate derivatives of various small Asp-containing peptides are given in Table I. It is clear from these data that a specific hydroxamate-induced cleavage is occurring, but that the cleavage does not go to completion. We shall return to this important point below. Furthermore, the maximum percent cleavage varies with the sequence.

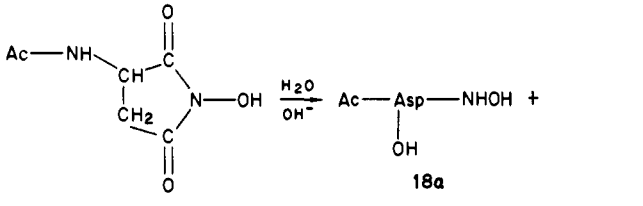
We sought to investigate whether the hydroxamate oxygen or nitrogen is the nucleophilic atom in the cleavage. The pK_a values for the following derivatives, determined by titration in water at room temperature, suggest that the conjugate base of the free hydroxamic acid group may be a mixture of both the N- and O-anions **15** and **16**.



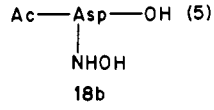
Indeed, the structure of hydroxamate anions has been controversial[10] for some time. The pK_a data above suggest, however, that both the nitrogen and the oxygen anions are available in

reasonable concentration under the conditions of our experiments. The results from cleavage of various methylated peptide hydroxamates are also given in Table I. The maximum percent cleavage is considerably lower in these peptides than in peptide **6**, possibly because of the steric effect of the methyl groups. Nevertheless, these data are consistent with the idea that at pH 6–7 both the nitrogen and the oxygen can act as nucleophiles.

Another approach to the same problem is to consider the possible products that would result from attack of each of the two potentially nucleophilic atoms. If the hydroxamate nitrogen is the nucleophile, subsequent opening of the resulting *N*-hydroxyimide **17** will give both α- and β-hydroxamates **18a** and **18b** as products. On the other hand, if the hydroxamate oxygen acts

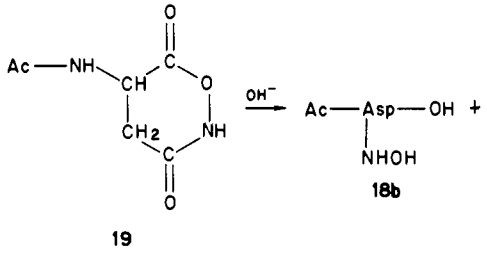


17 **18a**



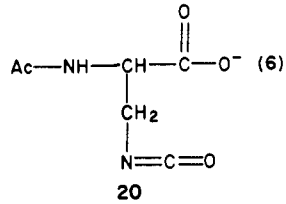
18b

as the nucleophile, the resulting perhydro-1,2-oxazine-3,6-dione derivative **19** would react with water only at one carbon to give the β-hydroxamate **18b**. Furthermore, since **19** is an *O*-acylhydroxamic acid, we would expect some Lossen rearrangement of this compound.[11] (Attempts to prepare compounds of this type were unsuccessful in our hands.) The result of such a rearrangement would be N^2-acetyl-2,3-diaminopropanoic acid, or a derivative (if the isocyanate **20** is trapped by other nucleophiles in solution). It is also possible that compound **19** could undergo

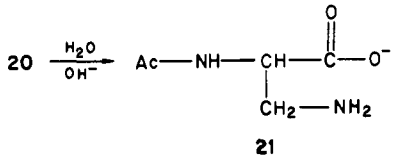


18b

19



20



21

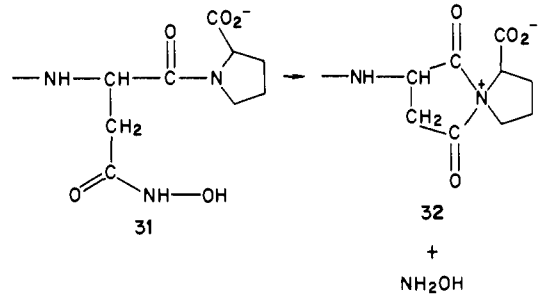
an O → N acyl shift; to the extent that this occurs, attack of oxygen and attack of nitrogen cannot be distinguished by product studies.

Reaction mixtures from the cleavages of peptides **6**, **11**, **12**, and **14** at 60 °C and pH 9.9, 10.0, 9.8, and 9.8, respectively, were examined by thin-layer electrophoresis at pH 4.1 (at which pH authentic samples of the various products of cleavage are separated). *Both* α- and β-hydroxamates **18a** and **18b** were clearly identifiable in these reaction mixtures. This result indicates that at least part of the desired specific cleavage must involve nucleophilic attack of the hydroxamate nitrogen and/or an O → N acyl shift of compound **19** at some stage in the reaction. Acid hydrolysis of the reaction mixtures from cleavage of peptides **6**, **10**, and **13** followed by amino acid analysis showed only 81%, 87%, and 86% recovery of aspartic acid, respectively. Hydrolysis in base[11] of the reaction mixture from cleavage of **13**, followed by amino acid analysis, gave 93% total recovery of Asp (as the sum of Asp and 2,3-diaminopropanoic acid) and revealed the presence of the latter (cf. **21**). These results clearly suggest that the hydroxamate oxygen also acts in a nucleophilic capacity at some point in the observed cleavage.

The data for unalkylated hydroxamates at different pH values in Table I show that the rate of cleavage does not vary markedly with pH, and only in the case of peptides **13** and **14** does the extent of cleavage vary significantly. The pH values in this table bracket the pK_a of the side-chain hydroxamate. If the ionization of this group were the only kinetically significant effect of pH, we should expect at least a 100-fold variation in rate over this pH range instead of the 4- to 7-fold variation observed. This analysis appears to rule out attack of the ionized hydroxamate as the rate-limiting step of the cleavage reaction. We shall return to this point below.

A mechanism that accounts for our results, as well as the results of the Bornstein and Balian cleavage, is shown in Scheme I. Attack of the hydroxylamine nitrogen (K_C) or oxygen (K_C') gives a tetrahedral addition intermediate **23** or **24** that breaks down with expulsion of peptide nitrogen, and a net cleavage results. A competing reaction is attack of the peptide amide nitrogen (K_N) on the hydroxamate carbonyl to give a tetrahedral adduct **25** that expels hydroxylamine to produce imide **28**. This imide can be attacked at either carbonyl group by water to give a mixture of α- and β-peptides **30a** and **30b**. Following the cleavage reactions of peptides **6**, **11**, and **12**, a mixture of the corresponding α- and β-peptides was identified (by electrophoresis at pH 4.1) in the cleavage reaction mixture; this result is reasonable only if hydrolysis of imide **28** occurs at either of its two carbonyl groups. To the extent that the K_N pathway is followed, cleavage is not observed, and this pathway accounts for the less than quantitative cleavage yield. Indeed, the peptide **14**, which incorporates the Asp-Gly sequence, shows a particularly low level of cleavage (Table I); this observation is in accord with the well-known tendency of Asp-Gly derivatives to undergo transpeptidation. The ultimate outcome of the K_N pathway is in fact a transpeptidation reaction.

Experiments with *N*-Ac-Asp-Pro, **31**, provided further evidence for the occurrence of the K_N pathway. We reasoned that the imide intermediate **32** generated from this peptide by intramolecular displacement of hydroxylamine would be less stable, and therefore it would be formed more slowly, because its formation requires the unfavorable development of positive charge on nitrogen adjacent to *two* carbonyl groups, i.e., a diacylammonium ion **32**. If



31 **32**

+

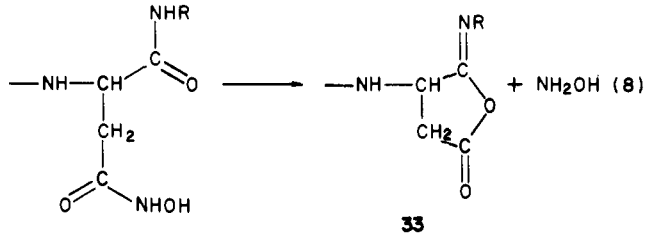
NH_2OH (7)

these suppositions are correct, then this side reaction should be relatively less important in the cleavage of **31**. Indeed, the cleavage yield of **31** (Table I) is larger than that of any other peptide; thus, it is clear that competing side reactions are relatively less important. The rates of cleavage of the proline derivative shown in Table I are not accelerated relative to the other peptides examined, and this observation is consistent with the notion that

(10) Bauer, L.; Exner, O. *Angew. Chem., Int. Ed. Engl.* **1974**, *13*, 376–384.
(11) Capecchi, J. T.; Miller, M. J.; Loudon, G. M. *J. Org. Chem.* **1983**, *48*, 2014–2021.

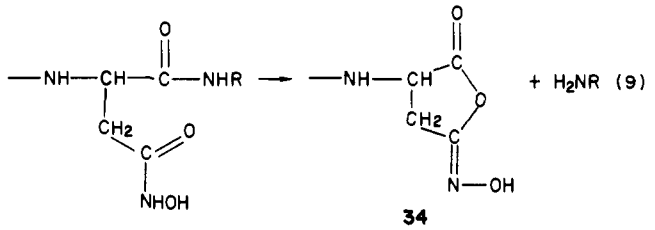
the improved yield is due to a selective reduction in the rate of the competing pathway. It is interesting, however, that even with the proline derivative **31** the cleavage yield is not quantitative. If the imide pathway is the source of the side reaction, then both α- and β-linked *N*-Ac-Asp-Pro isomers (analogous to **30a** and **30b**, Scheme I) should be formed by attack of water (or OH^-) at either carbonyl group of **32**. In fact, these materials were clearly demonstrated by thin-layer electrophoresis to be present in the reaction mixture along with compounds **29a** and **29b** (Scheme I).

Another possible family of mechanisms that cannot be neglected involves attack of the respective carbonyl oxygens to give isoimide, or iminoanhydride, intermediates. For example, corresponding to the K_N pathway is the reaction



33

and corresponding to the cleavage pathway K_C is the reaction shown in eq 9 Model studies in the literature clearly suggest that



34

under the conditions of our experiments the isoimides **33** and **34** will either hydrolyze at their carbonyl groups or undergo buffer-mediated isomerization to their respective imides **28** and **27**.[12] Whether imides are formed by isomerization of **33** and **34** or by the direct attack of amide nitrogen, significant involvement of these imides is indicated by the product studies cited above. The extent to which isoimides are involved as intermediates cannot be assessed from our data.

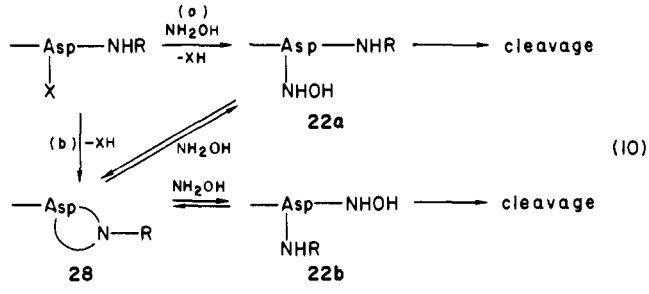
Cleavage of Peptides in the Presence of Excess Hydroxylamine. The reactions shown in Scheme I and eq 8 suggest a useful way to improve the cleavage yield. If excess hydroxylamine is incorporated into the reaction mixture, it should compete with water or OH^- for the imide **28** (dashed arrow—the β-isomer should also be formed in this reaction; similarly, isoimide **33** in eq 7 will also react with hydroxylamine). To the extent that this occurs, the competing pathway K_N can be reversed, and a greater fraction of the K_C (and K_C') pathways should be observed.

To test this hypothesis, the various peptides used in this study were subjected to cleavage reactions in the presence of 1 M hydroxylamine both at pH 6.4 (hydroxylamine buffer) and at pH 8–9. In order to minimize any specific buffer effects, no exogenous buffer was added at either pH. The pH of the solutions at the higher pH, initially about 8, drifted to about 9 during the course of the reaction. This drift may be a consequence of some decomposition of hydroxylamine to ammonia and other products in alkaline solution containing oxygen.[13] Because these reaction tubes were closed, they were protected from exogenous oxygen, so it is unlikely that significant decomposition of the hydroxylamine took place in these experiments. Nevertheless, a high degree of quantitative significance was not placed on the data obtained at the higher pH values. At pH 6.4 the solution pH was stable; hydroxylamine does not decompose at this pH to a significant extent.[13d] Kinetic data obtained at this pH showed that the cleavage reactions were accurately pseudo first order through at least 4 half-lives; this observation is one further indication that no significant decomposition of hydroxylamine is occurring at this pH. The fact that hydroxylamine under alkaline conditions promotes the oxidation of some proteins[13e] suggests, however, that when the cleavage described here is ultimately applied to such proteins it should be carried out at the lowest possible pH.

The cleavage results are given in Table II. The results in the presence and absence of hydroxylamine are contrasted in Tables II and Figure 1. It is clear that the 1 M hydroxylamine conditions substantially increase cleavage yield; in fact, quantitative cleavage is observed in several cases. Furthermore, authentic samples of the putative imide intermediates (compounds **34**, **35**, and **36**) undergo cleavage, as they must if the hypothesis above is correct. Results with most of the control peptides shown in the same table demonstrate that even in the presence of 1 M hydroxylamine the cleavage ramains specific for the side-chain hydroxamate group. One exception is the cleavage of Asn–Phe at pH 6.4, which probably occurs by way of direct hydroxylaminolysis of the amide side chain rather than imide formation; hydroxylaminolysis rates of amides are maximal at pH 6.[14] The Asn-Gly sequence also shows substantial cleavage in the presence of hydroxylamine, as expected.[6] Cleavage of the Asn-Gly sequence at pH 9 is considerably slower than that at the corresponding Asp(β-hydroxamic acid) sequence.

The rates and extent of cleavage in the presence of 1 M hydroxylamine, as in the absence of the added nucleophile, differ little at pH 6.4 and pH 8–9. The rate data (not shown) demonstrate that the rates differ by factors of only 3–9 at the two pH values. The lack of a substantial pH effect on the cleavage is similar to the same observation in the absence of hydroxylamine.

Compounds **8** and **9** of Table II show that hydroxylamine itself need not be the leaving group in the 1 M hydroxylamine cleavage. In these examples the cleavage yields of peptides containing *N*-methylhydroxamate or *O*-methylhydroxamate groups are increased by added hydroxylamine. Presumably other side-chain aspartyl derivatives, such as aspartyl esters, would also be cleaved specifically in the presence of 1 M hydroxylamine. This cleavage could take place by either (or both) of two mechanisms, shown in eq 10. In mechanism (a), hydroxylamine displaces the leaving



28 **22a** **22b** (10)

group X at the side-chain aspartyl carbonyl to give the hydroxamic acid **22a**, which then cleaves by the mechanisms discussed above. In another mechanism, (b), an imide **28** (Scheme I), formed under the basic conditions of hydroxylaminolysis, is intercepted to form β- and α-hydroxamates **22a** and **22b**, which also cleave by the hydroxamate mechanisms discussed above.

Acid hydrolysis of the cleavage reaction mixtures, followed by amino acid analysis, shows no loss of Asp in the presence of 1 M hydroxylamine. Evidently any initially formed perhydro-1,2-oxazine-3,6-dione (**26**) (Scheme I) is intercepted by the added hydroxylamine before it can undergo the Lossen rearrangement.

(12) (a) Ernst, M. L.; Schmir, G. L. *J. Am. Chem. Soc.* **1966**, *88*, 5001–5009. (b) Paul, R.; Kende, A. S. *J. Am. Chem. Soc.* **1964**, *86*, 4162–4166. (c) Sauers, C. K.; Marikakis, C. A.; Lupton, M. A. *J. Am. Chem. Soc.* **1973**, *95*, 6792–6799. (d) Boyd, G. V.; Monteil, R. L. *J. Chem. Soc., Perkin Trans. 1* **1978**, 1338–1350.

(13) (a) Moews, P. C.; Audrieth, L. F. *J. Inorg. Nucl. Chem.* **1959**, *11*, 242–246. (b) Yagil, G.; Anbar, M. *Ibid.* **1964**, *26*, 453–460. (c) Hughes, M. N.; Nicklin, H. G. *Chem. Ind. (London)* **1967**, 2176. (d) Anderson, J. H. *Analyst* **1964**, *89*, 357–362. (e) Kaseman, D. S.; Meister, A. "Abstracts, 186th National Meeting of the American Chemical Society", American Chemical Society: Washington, DC, 1983; Biol. Chem. No. 11.

(14) Jencks, W. P.; Gilchrist, M. *J. Am. Chem. Soc.* **1964**, *86*, 5616–5620.

Table II. Effect of 1 M Hydroxylamine on the Specific Cleavage at 60 °C of Peptides Containing Side-Chain Hydroxamate Groups[a]

compd no.	peptide	+1 M hydroxylamine; max % cleavage at pH 8–9	+1 M hydroxylamine; max % cleavage at pH 6.4	no hydroxylamine;[b] max % cleavage at pH 9–10	no hydroxylamine;[b] max % cleavage at pH 6–7
6	*N*-Ac-Asp-Ala (NHOH)	100	96	65 (9.5)	57 (6.8)
8	*N*-Ac-Asp-Ala (NH—OCH_3)	80	88	8 (9.5)	30 (6.8)
9	*N*-Ac-Asp-Ala ($N(CH_3)OH$)	90	>80	0 (9.5)	20 (6.8)
11	*N*-Ac-Asp-Phe (NHOH)	89	81	71 (9.9)	67 (6.9)
12	*N*-Ac-Asp-Ala-Gly (NHOH)	74	86	20 (9.5)	21 (6.7)
13	*N*-Ac-D,L-Asp-Val-Ala (NHOH)		65	60 (9.4)	38 (6.8)
14	*N*-Ac-Asp-Gly (NHOH)		84	16 (9.5)	53 (6.8)
35	*N*-Ac-D,L-Aim[c]-Gly	100	92	0 (9.4)	0 (6.8)
36	*N*-Ac-Aim[c]-Ala		83	0 (9.4)	0 (6.8)
37	*N*-Ac-Aim[c]-Phe		65	0 (9.4)	0 (6.8)
	control peptides[d]				
38	*N*-Ac-Asp-Ala	0	0	0 (9.7)	0 (6.8)
39	*N*-Ac-Asn-Phe	10	38	2 (9.7)	0 (6.8)
40	*N*-Ac-Asn-Gly	42	11	0 (9.5)	0 (6.8)
41	*N*-Ac-Asp-Ala-Gly	0	6	0 (10.0)	0 (6.9)
42	*N*-Ac-Asp-Phe	0	0	0 (9.5)	0 (6.8)
43	*N*-Ac-Asp-Gly	0–2	4–8	0 (9.5)	0 (6.8)

[a] At pH 6.4, 0.1 M NaH_2PO_4 buffers were used. At pH 8–9, pH is established as discussed in the text. [b] Data at pH 9–10 from Table I. The measured pH value is given in parentheses. [c] Aim = aspartimide. [d] Percent completion of the control peptide is the amount of Asp-X cleavage observed when cleavage of the corresponding hydroxamic acid containing peptide has proceeded for 4 half-lives.

The mechanistic analysis above suggests that the role of hydroxylamine is to trap imide **28** in Scheme I and divert it back to productive pathways. The cleavages of imides **35**, **36**, and **37** are necessary but not sufficient to demonstrate that this pathway is viable. If the intermediacy of the imide is to account for the greater fraction of cleavage observed in the presence of added hydroxylamine, then when examined independently it should also be cleaved at least as rapidly as the starting peptide itself. Were it to be cleaved more slowly, it would be ruled out as a significant intermediate. Kinetic data for cleavage of imides are given in Table III.

It appears from these data that the cleavage of imide, at least in the case of peptides **6** and **14**, cannot account kinetically for more than a small fraction of the observed rate of reaction. In the case of peptide **11** the imide is marginally competent as an intermediate. However, in the hydroxylaminolysis of this peptide the kinetics were accurately first order and gave no evidence for the accumulation of an intermediate. Since the hydroxylaminolysis of imide **37** occurs at a rate very similar to that of the parent peptide **11**, it might be expected that the imide, if a significant intermediate, would accumulate in the reaction mixture. Of course the kinetic analysis would not detect small amounts of imide buildup. It is also possible that isoimides (**33**, eq 8) are the major nonproductive intermediates, and it is the interception of these by hydroxylamine that accounts for the results. Since isoimides are about 200 times more reactive than imides at pH 6.5 toward hydrolysis,[12a] there seems to be no question that these intermediates would be kinetically competent. There is, however, one additional effect that may be operating to increase the cleavage yield in the presence of hydroxylamine: the selective catalysis of the cleavage pathway by hydroxylamine (or its conjugate acid) acting as a general acid–base catalyst.

Effect of Buffer Catalysis. Another possibility for the role of hydroxylamine is simply that it, as a general base, promotes cleavage by selectively catalyzing breakdown of the tetrahedral intermediates **23** or **24** (Scheme I). It has been shown in several instances that the rate-limiting step of many acyl-transfer reactions is diffusion-limited proton transfer to or from buffer bases and acids, and furthermore that such buffer catalysis can markedly influence product ratios.[15] Accordingly, we examined the effect of buffer (at pH 6.4–6.5) on the cleavage yield for the Asp-Ala-Gly derivative **12**. The data are presented in Table IV. The pseudo-first-order rate constant for cleavage, k_C, can be obtained by multiplying the observed pseudo-first-order rate constant for disappearance of starting material, k_{obsd}, by the maximum percent cleavage; and the rate constant for pathways other than cleavage, k_N, is the difference between k_{obsd} and k_C.[16] In Figure 2 is shown

Table III. Specific Cleavage of Imides and the Corresponding Peptides at 60 °C and pH 6.4[a]

compd no.	peptide	max % cleavage	$10^5 k_C$,[b] s^{-1}
35	*N*-Ac-D,L-aspartimide-Gly	92	3.6
14	*N*-Ac-Asp-Gly (NHOH)	84	17.
36	*N*-Ac-aspartimide-Ala	83	3.4
6	*N*-Ac-Asp-Ala (NHOH)	96	5.8
37	*N*-Ac-aspartimide-Phe	65	2.2
11	*N*-Ac-Asp-Phe (NHOH)	81	2.2

[a] 1 M hydroxylamine buffers. [b] Rate constant for cleavage is k_{obsd} times the maximum fraction cleavage.[16]

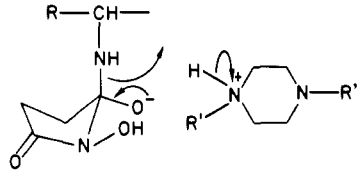
(15) (a) Satterthwait, A. C.; Jencks, W. P. *J. Am. Chem. Soc.* **1974**, *96*, 7031–7044. (b) Smith, V. F.; Jr.; Schmir, G. L. *J. Am. Chem. Soc.* **1975**, *97*, 3171–3177. (c) Jencks, W. P. *Acc. Chem. Res.* **1976**, *9*, 425–432. (d) Cox, M. M.; Jencks, W. P. *J. Am. Chem. Soc.* **1981**, *103*, 580–587. (e) Lee, Y.-N.; Schmir, G. L. *J. Am. Chem. Soc.* **1979**, *101*, 3026–3055, 6277–6282. (f) Cunningham, B. A.; Schmir, G. L. *J. Am. Chem. Soc.* **1966**, *88*, 551–558; **1967**, *89*, 917–922.

Table IV. Buffer Catalysis in the Cleavage of *N*-Ac-Asp(β-hydroxamic acid)-Ala-Gly (**12**) at 60 °C

pH	buffer,[a] concn (*M*)	$10^5 k_{obsd}$,[b] s^{-1}	max % cleavage
6.40	NH_2OH·HCl, 1.0	6.3	84
6.4	NaH_2PO_4, 0.1	6.0	30
6.4	NaH_2PO_4, 0.5	7.8	41
6.5	NaH_2PO_4, 1.0	11.4	59
6.33	PIPES, 0.04	1.7	37
6.38	PIPES, 0.08	2.0	36
6.30	PIPES, 0.42	2.0	24
6.41	PIPES, 0.84	2.5	23
6.33	imidazole·HCl, 0.05	2.7	30
6.35	imidazole·HCl, 0.10	3.9	28
6.35	imidazole·HCl, 0.50	7.8	28
6.48	imidazole·HCl, 1.00	9.7	35

[a] Acidic form of the buffer given; ionic strength was maintained at 2.0 M with KCl. Except for the first entry, hydroxylamine was absent. PIPES is 1,4-piperazinebis(ethanesulfonic acid). [b] Observed rate constant; the rate constant for cleavage, k_C, is calculated by multiplying k_{obsd} by the maximum fraction cleavage;[16] the rate constant for competing pathways, k_N, is the difference between k_{obsd} and k_C. The constants k_C and k_N are shown in Figure 2, a and b, respectively.

a plot of both k_C and k_N vs. total buffer concentration. It is clear from Table IV that certain buffers can significantly affect the fraction of cleavage observed. The enhancement of cleavage yield by phosphate clearly occurs because catalysis of cleavage is a linear function of phosphate, whereas the competing pathways are not a function of phosphate over the range of buffer examined. Imidazole buffers catalyze both cleavage and hydroxylamine release, with the result that only a very modest increase, if any, of cleavage yield is observed. PIPES (1,4-piperazinebis(ethanesulfonic acid)) buffers slightly reduce the cleavage yield, and evidently they do so because competing pathways are modestly catalyzed, whereas cleavage is not. The reasons for these differential buffer effects cannot be determined with certainty from the data available, but it may be significant that phosphate has been shown to be particularly effective as a bifunctional catalyst.[15] In imidate ester hydrolysis it selectively catalyzes the release of the more basic leaving group (that is, amine rather than alcohol) from the tetrahedral addition intermediate. It is also worth noting that the data at pH 9–10 in the absence of hydroxylamine (Table I, Figure 1) were obtained in the presence of a bicarbonate buffer, and bicarbonate is also known to be an effective bifunctional catalyst.[15b] Furthermore, Bornstein and Balian[6] found the optimum conditions for cleavage of ribonuclease to be 2 M hydroxylamine in a bicarbonate buffer. The absence of a strong catalytic effect of PIPES may be due to a steric effect: perhaps the sterically congested nitrogen of PIPES has difficulty in approaching the rather congested tetrahedral intermediate leading to cleavage; that is, the energy of transition state **44** is raised by nonbonded repulsions.



44

On the reasonable assumption that at zero buffer concentration the buffer plots in Figure 2 should approach the same value, it appears that there is significant curvature to the buffer plots in Figure 2b, but not in Figure 2a. Such curvature could indicate a change of rate-determining step, and therefore a kinetically significant intermediate, in the competing pathways. The precise reason for this curvature, however, cannot be determined from the data available.

(16) Moore, J. W.; Pearson, R. G. "Kinetics and Mechanism", 3rd ed.; Wiley: New York, 1981; pp 284–286.

The buffer catalysis of the cleavage reaction is superimposed on any catalytic effects of lyate species (OH^-, H_2O). If buffer catalysis is much more important than lyate catalysis, and if some or all of the proton transfers from buffers to tetrahedral intermediates are diffusion controlled in the thermodynamically favorable direction,[15c] then the catalytic effects of buffers might show little dependence on buffer pK_a, and as a result, there would be relatively little apparent variation of the reaction rate with pH, as observed (Table I). A complete analysis of lyate vs. buffer catalysis would require a detailed study of rates and product distributions as a function of both pH and buffer that are outside the scope of this study.

Clearly certain buffers can catalyze the partitioning of the reaction into the cleavage mode. It is thus possible that much of the effect of added hydroxylamine shown in Table II is a buffer effect. In other words, it is possible that hydroxylamine may increase cleavage yield not only by acting as a nucleophile and intercepting the imide **28** or isoimide **33** but also by selectively catalyzing the breakdown of tetrahedral intermediates **23** and **24**. If hydroxylamine is acting as a buffer catalyst, then it must catalyze the reaction—that is, a peptide hydroxamate must show a cleavage rate considerably greater than the rate expected in the absence of hydroxylamine or other buffer. The asterisk in Figure 2a represents the rate of cleavage at 1 M hydroxylamine; that this rate is considerably greater than the rate extrapolated to zero buffer supports the notion that hydroxylamine is actually catalyzing the cleavage reaction. It is also worth noting that if the k_N process represents formation of imide **28** (Scheme I) or isoimide **33** (eq 8), catalysis of this process by hydroxylamine would be more nearly "invisible", since when these species are formed they would be opened almost every time by hydroxylamine to regenerate the α- and/or β-isomers of the starting peptide hydroxamic acid, which can be cleaved. If this analysis is correct, the rate for the k_N pathway in the presence of 1 M hydroxylamine represents the residual lyate (water + OH^-) attack on these hydrolyzable intermediates. It is interesting, although perhaps coincidental, that the value of k_N (asterisk, Figure 2b) is essentially identical with the value for k_N obtained at zero buffer.

Cleavage of Glutamyl Peptides. If hydroxamic acid groups can be introduced into aspartic acid residues of peptides or proteins by an appropriate modification technique, it would be expected that glutamic acid residues would also be modified and converted into their hydroxamic acids. Thus it is important to examine the cleavage of peptides containing glutamyl(γ-hydroxyamic acid) residues and compare the rates of their cleavage reactions with those of aspartyl(β-hydroxamic acid)-containing peptides. The side-chain hydroxamates of Ac-Glu-Ala and Ac-Glu-Ala-Gly were prepared and subjected to cleavage conditions at both pH 6.5 and 8–9 in the presence of 1 M hydroxylamine at 60 °C. In both cases, cleavage of these peptides occurred to the extent of only 6–10% when the analogous aspartyl peptides had undergone cleavage for 4 half-lives. It is estimated that peptides containing aspartyl(β-hydroxamic acid) residues cleave 20–40 times faster than the analogous glutamyl(γ-hydroxamic acid)-containing peptides. This finding is completely in accord with literature data on the relative ease of neighboring group participation of the amide nitrogen through six- vs. five-membered cyclic transition states.[8a,b,h]

Conclusions. It is clear from this work that the general mechanism of the Bornstein–Balian Asn–Gly cleavage[6] shown in eq 2 is supported by the data. Of perhaps more practical importance is the fact that if side-chain hydroxamate groups can be introduced into peptides by appropriate modification techniques a specific cleavage at all aspartic acid residues so modified will be possible. Such protein modifications are the subject of ongoing studies in our laboratory.

Experimental Section

Reagents, peptides, and amino acid derivatives were purchased from Sigma, Aldrich, Vega Biochemicals, or Pierce Chemical Co. Amino acid analyses were performed on a Beckman 119CL Amino Acid Analyzer and are corrected to an internal standard. The ^1H nuclear magnetic resonance (NMR) spectra were recorded either on a Varian Associates FT-80 (80 MHz) or a EM-360 spectrophotometer (60 MHz) with either

tetramethylsilane (Me_4Si) or sodium 3-(trimethylsilyl)-1-propanesulfonate (DSS) as the internal standard; Me_2SO-d_6 was used as the NMR solvent unless otherwise noted. Infrared (IR) spectra were obtained with a Beckman IR-33 grating spectrophotometer.

All column chromatography was done on gravity flow columns. Anion exchange resins, DEAE-Sephadex and Dowex AG1-X_2 (Bio-Rad), were always used in the acetate form. Cation exchange resins, Dowex AG50W-X_2 and AG50W-X_4 (Bio-Rad), were always used in the proton form. Unless noted otherwise, the buffer gradient used was a linear one prepared from water and 1 M acetic acid adjusted to pH 3.9 with pyridine.

Thin-layer electrophoresis (TLE) was run on a Camag High Voltage Electrophoresis System at 900 V for 40 min (unless otherwise indicated) with Eastman cellulose plates (No. 13255) and a buffer prepared from 0.52 M acetic acid adjusted to pH 4.1 with pyridine. All relative mobilities, R_m, are reported relative to a picric acid marker. Thin-layer chromatography (TLC) was performed on Merck (No. 5775) silica gel plates. Preparative TLC was run on either Merck 2 or 0.25 mm precoated silica gel plates (No. 5776 and No. 5765, respectively).

Titrations were made on a Metrohm Combi-Titrator E512/E473/E425 instrument. Optical rotations were taken on a Perkin-Elmer Model 241 polarimeter (1 mL, 1.0001 dm, jacketed cell). Conductivity measurements were made with a London-type CDM2e conductivity meter. Melting points were taken on a Büchi (oil immersion) melting point apparatus and are uncorrected. Elemental analyses were performed by the Microanalytical Laboratory of the Purdue University Chemistry Department.

General Procedure for Peptide Cleavage Reactions. A peptide sample of known weight (10–40 μmol) in a 15-mL Nalgene tube was dissolved in an appropriate buffer such that 4.9 mL of solution was produced. A 0.1-mL aliquot of an internal standard solution (a nonproduct amino acid or norleucine) was added to produce a final volume of 5.0 mL (final concentration = 2–8 mM in starting peptides). The Nalgene tube was then fitted with a rubber septum and the tube contents mixed well on a Vortex mixer. The tube was placed in a stirring oil bath at 60 °C and, if an accurate weight of the peptide had not been obtained, the exact reaction concentration was determined by removal of a 0.1-mL aliquot and subsequent hydrolysis/amino acid analysis. This value, whether calculated or determined by amino acid analysis and corrected to the internal standard, served as the amount of product required for 100% completion of cleavage. Cleavage reaction progress was determined as follows. At various times after the reaction tube entered the oil bath, 0.1-mL reaction aliquots were removed and diluted to ca. 1 mL by the addition of 0.9 mL of pH 2.2 sodium citrate diluting buffer at 4 °C. This procedure was found to stop effectively any cleavage process and not to promote nonspecific cleavage. A 0.1-mL aliquot of this solution was then placed on the amino acid analyzer and the cleavage product (an amino acid or dipeptide) quantitated by peak integration. The accuracy of all integrations is ±5%. Dilution or evaporation errors were corrected by the presence of the internal standard. The percent completion of cleavage at any time was determined by dividing the quantitated amount of cleavage product by the amount required for 100% reaction.

The buffers used in most of the cleavage experiments are described in the appropriate tables. Hydroxylamine buffers at pH 7–9 were initially prepared by dissolving the amount of hydroxylamine hydrochloride required for a final concentration of 1 M in a 15-mL Nalgene tube containing 2.5 mL of 0.05 M carbonate–bicarbonate buffer. To obtain a 1 M hydroxylamine buffer at a pH near 7, 6.25 M NaOH was added dropwise to the appropriate amount of NH_2OH·HCl until the desired pH was reached and then the solution was diluted to the proper volume with water. A solution of 1 M hydroxylamine at pH 8–**9** (not a buffer) was prepared in exactly the same fashion except that the NH_2OH·HCl was completely neutralized by the addition of 1 equiv of NaOH. Preparing the buffers in this way became impossible when, on one occasion, all of the NH_2OH·HCl precipitated out of solution as the pH was increased. Precipitation was then observed to occur on every subsequent attempt to prepare the buffers in this fashion. This problem was overcome by using water (not carbonate–bicarbonate buffer) to dissolve the NH_2OH·HCl and very clean glassware (not Nalgene). There was no noticeable effect on the outcome of the cleavage experiments as a result of this change.

The pH values cited in this paper are those actually measured at 60 °C unless otherwise indicated. Cleavage reaction mixtures prepared with dilute inorganic buffers showed very little dependence of pH on temperature and were effectively buffered even at long reaction times (less than 0.2 pH unit change even after 5 days of reaction). The hydroxylamine buffer prepared at pH 7 (room temperature) had an average solution pH of 6.54 at 60 °C and was also an effective buffer (less than 0.2 pH unit change after 2 days of reaction). Buffering with hydroxylamine at pH 9 was impossible since its pK_a value is 6.03. Cleavage reactions prepared with a pH **9** solution of 1 M hydroxylamine gave initial pH values of ca. 8.1 at 60 °C. After 2 days of reaction this had increased to ca. 9. Although a different buffer could have been used and the hydroxylamine still included, this was not done so that additional variables would not be introduced into the cleavage procedures. The pH of these poorly buffered hydroxylamine solutions is cited as pH 8–**9**, and it is meant to be indicative of the approximate initial and final measured pH values.

Hydrolysis of Peptides. Peptide samples subjected to acid hydrolysis prior to amino acid analysis were incubated in 6 N HCl for 24 h at 110 °C in evacuated sealed tubes. For hydrolysis of peptides in base, a modification of the method of Capecchi et al.[11] was used. Following the completion of an individual cleavage reaction and the determination of Asp by acid hydrolysis, all of the solvent was removed in vacuo. Extensive degassing on a water aspirator was required before high vacuum could be used in order to prevent severe bumping of the solution. The appropriate amount of $Ba(OH)_2$·$6H_2O$ (to give a 5–6 N solution) was then added to the cleavage reaction concentrate and then enough freshly boiled water was added to achieve the desired volume. The Nalgene tube was then fitted with a rubber septum and the tube contents mixed well on a Vortex mixer. This was placed in a stirred oil bath at 110 °C for 24 h. A 0.1–0.3 mL aliquot of the hot reaction solution was then removed with a disposable pipet (as rapidly as possible to prevent precipitation of $Ba(OH)_2$), diluted with 1 equiv of acid (HCl), and then further diluted with pH 2.2 sodium citrate, diluting buffer to the desired volume. A sample of this solution was then applied directly to the amino acid analyzer, and the ninhydrin-reactive products were quantitated.

Electrophoretic Analysis Procedure. Following the completion of the cleavage reaction, all of the solvent was removed in vacuo. The resulting concentrates were then diluted to 0.1–0.2 mL total volume with water to give a final concentration of 30–100 mM in cleavage product. Peptide standard solutions were prepared at a concentration of 60–100 mM in water. Samples of the cleavage reaction concentrates (3 μL) and appropriate peptide standard solutions (1 μL) were applied to cellulose TLE plates. After electrophoresis the plates were dried well at room temperature and the products visualized with $FeCl_3$ (3% in 0.5 M HCl)[17] or ninhydrin and Cl_2/starch:KI[18] stains. If $FeCl_3$ stain was used, the TLE plate could be sprayed with this reagent after only a very cursory drying. Effective ninhydrin and Cl_2/starch:KI staining, however, required that the plate be air-dried for at least 24 h. Relative mobilities (R_m) of all product spots vs. picric acid were then determined.

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Supplementary Material Available: Synthetic procedures (23 pages). Ordering information is given on any current masthead page.

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