may be therapy-limiting. Our mechanisms suggest that SoSo and BCNU produce cross-linked base pairs with different regioselectivities, the structures of which may account for differential repair phenomena.

There is evidence, however, that DNA cross-linking may not be a primary cytotoxic mechanism in some, often viral-transformed, cell lines. Gibson in particular has found that alkylation of BE cell DNA with MeSoSo, and less so with EtSoSo, causes almost as much cell kill as treatment with the chloroethyl compound.[58] In these and possibly in other cell lines, regioselective alkylation may be an important cytotoxic mechanism or may be responsible for other events. Seidenfeld's results for induction of heat shock enzymes by CENU treatment of BE cells may be related to proximity formation of carbamoyl (or urea) and alkyl adducts in cells.[41]

It is clear that the cytotoxic mechanisms of these and other anticancer drug are complex. SoSo and BCNU are two of the structurally *simple* compounds that are used clinically for the treatment of human neoplasia. Despite a great deal of often elegant work, we are only now beginning to have a vague understanding of mechanisms of action of these and other anticancer drugs and of mechanisms of DNA alkylation that lead to mutagenic and carcinogenic transformations in situ.

Experimental Section

Determination of the Percent Cross-Linking of CT-DNA. For DNA treatment, BCNU (10–20 mM) and HN2 (0.2 mM) were dissolved in absolute ethanol and SoSo (20–40 mM) was dissolved in dimethyl sulfoxide. CT-DNA (200 μg/mL in 50 mM potassium phosphate buffer, pH 7.2) was either treated continuously (no drug removal) or pulse treated with the drugs for up to 1 h (drug-absent protocol). In the latter, unreacted drug was removed by precipitating DNA with 2.5 volumes of cold (−20 °C) ethanol, washing the precipitate with ice-cold 80% ethanol, and redissolving precipitate in fresh warm buffer, which was incubated at either 37 ± 0.2 or 50 ± 0.2 °C in a thermostated water bath.

The percent cross-linking was determined by using the method of Morgan and Paetican.[49] Briefly, about 10 μg of treated DNA in buffer was added to 3 mL of a solution of ethidium bromide (1 μg/mL), 0.4 mM EDTA, and 20 mM potassium phosphate (pH 11.8). Fluorescence was measured in 1-cm² cuvettes at ambient temperature in a Perkin-Elmer LS-F fluorescence spectrophotometer using 525-nm excitation and 600-nm emission wavelengths. The solution was heated to 100 °C for 5 min. After the solution was cooled rapidly in ice water, the fluorescence was measured again. The fraction of the original fluorescence retained is the fraction of DNA cross-linked.

Kezdy-Swinbourne Method.[51] Values of X_∞ were determined from plots of X_t vs $X_{t+\tau}$ where τ is an interval between datum points generally chosen to equal 1.5–2 half-lives. Slopes were calculated by linear regression for various values of τ, and the slope with the best correlation coefficient was used. In general, larger values of τ give more accurate estimates of X_∞, and we chose the largest value of τ consistent with good plots. Values of τ on the order of 24–27 h are available from the precursor formation data. X_∞ was estimated graphically by constructing lines with the first datum point and the calculated slope; the intersection of this line with the 45° line is X_∞. For small or large values of τ, k_{obsd} calculated with either the slopes of Kezdy-Swinbourne plots or the estimated X_∞ values were generally within ±10%, but deviations of as much as 20% were found at the lower temperature and lower concentrations used. Rate constants can be calculated directly from slopes of Kezdy-Swinbourne plots with the equation $k_{K\text{-}S}$ = (ln slope)/τ; values are listed in Table I.

Acknowledgment. Supported in part by NIH Program Project Grant CA-13525, the Department of Neurological Surgery, UCSF, and the Phi Beta Psi Sorority (N.B.) and by NIH Grants CA-14799, CA-36888, and CA-21765, and by the American Lebanese Syrian Associated Charities (T.P.B.). We gratefully acknowledge the excellent technical assistance of Joanna Remak (St. Jude's). The stereodiagrams are from work done by N.B. with Burt G. Feuerstein and Laurence J. Marton, who kindly allowed us to use them here. We thank Neil W. Gibson, Robert F. Struck, Jerome Seidenfeld, Anthony Pegg, and Barry Gold for allowing us to cite unpublished data or for providing preprints or galleys; Richard H. Shafer, Norman Oppenheimer, Hirak Basu, and Neville Kallenbach for helpful discussions; John Cashman for comments on an early draft of the manuscript; and Robert Landridge for use of the MIDAS and AMBER programs and the UCSF Computer Graphics Laboratory (supported in part by NIH Grant RR 1081). Finally, two referees caught a critical error, for which we are most grateful.

Registry No. BCNU, 154-93-8; SoSo, 88343-72-0.

(77) Brent, T. P.; Smith, D. G.; Remack, J. S. *Biochem. Biophys. Res. Commun.* **1987**, *142*, 341–347. Brent, T. P.; Remack, J. S.; Smith, D. G. *Cancer Res.* **1987**, *47*, 6185–6188. SoSo precursor adducts also react with GATase but at a slower rate than found for BCNU.

Hydrolysis of a Peptide Bond in Neutral Water

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Contribution from the Department of Chemistry, Columbia University, New York, New York 10027. *Received April 4, 1988*

Abstract: A radioassay for measuring amide bond hydrolysis at neutral pH and room temperature is presented. A peptide labeled at the terminal amino acid is attached to a polyacrylamide resin. The derivatized resin is incubated in buffer, and the release of radiolabel into bulk solution is monitored as a function of time. These data are used to calculate a rate constant for the process leading to radiolabel release. Control experiments indicate that this process is amide bond hydrolysis. The rate of hydrolysis of resin-PhePhePheGly at neutral pH and room temperature was found to be 3×10^{-9} s^{-1}, which corresponds to a half-life of approximately 7 years.

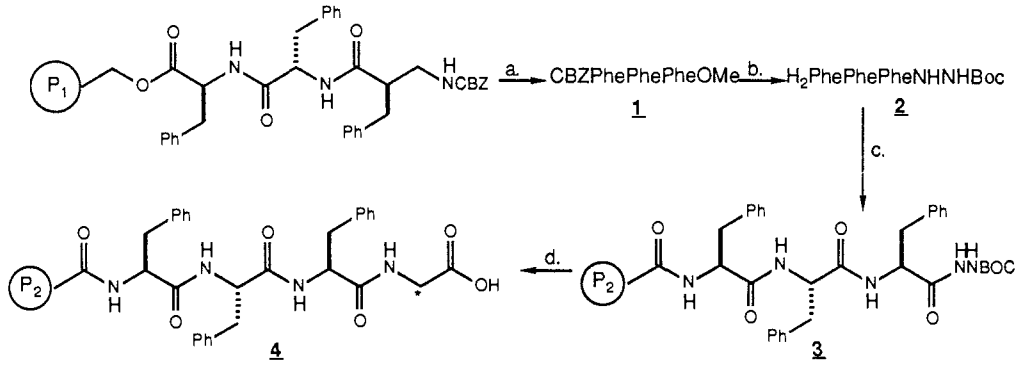
Simple hydrolysis of an unactivated amide has never been observed in pure water. What is known about the rate of amide hydrolysis under neutral conditions has been determined either by inference from the properties of more reactive functionalities, such as esters or amides activated by conjugation, or by extrapolation of results from amide hydrolyses studied under more vigorous conditions. Perhaps the mildest conditions reported for hydrolysis of an unactivated amide are those used by Meriwether and Westheimer.[1] They established the rate of glycinamide hydrolysis to be 2×10^{-6} s^{-1} at pH 9.3 and 65 °C.

Our interest in the hydrolysis of amides stems from ongoing work here which is directed toward the construction of a semisynthetic peptidase for the selective hydrolysis of certain C-terminal peptides. The design of artificial enzymes has been the focus

(1) Meriwether, L.; Westheimer, F. H. *J. Am. Chem. Soc.* **1956**, *78*, 5119.

0002-7863/88/1510-7529$01.50/0

Scheme I[a]



P_1 = Merrifield Resin, P_2 = Sheppard Resin

[a] (a) MeOH/Et_3N, 5 days, 89%; (b) (1) N_2H_2/DMF, 30 min; (2) BOC anhydride/*N,N*-diisopropylethylamine/DMF, 4 h, 93%; (3) Pd/$BaSO_4$/MeOH/H_2, 89%; (c) Polyacrylamide resin acyl azide/DMF, 5 °C for 12 h, room temperature for 24 h, 79%; (d) (1) HCl/HOAc (1:9), 30 min; (2) BuONO/DMF/H_2O, −5 °C, 30 min; (3) [2-^{14}C]Gly/H_2O/DMF, −5 °C, 36 h.

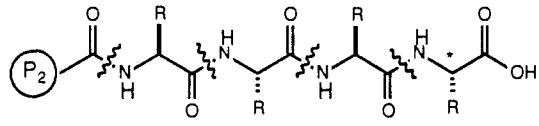


Figure 1. Radiolabeled polymer-bound amide.

of intense effort for a number of years, and while progress in the hydrolysis of relatively labile amide surrogates such as nitrophenyl esters has been noteworthy, no report of a functioning artificial peptidase has yet appeared.[2] One reason why true artificial peptidase activity has not yet been observed may be that the available assays have not been sensitive enough to detect modest rate enhancements in the hydrolysis of unactivated amides.

In order to detect small changes in the rate of amide hydrolysis, one would ideally like to measure the basal rate of hydrolysis in the medium being used. In this paper we describe a radioassay capable of detecting the hydrolysis of the C-terminal glycine amide linkage of the polymer-bound tetrapeptide RCOPhePhePheGlyOH in pure water at 25 °C. The pseudo-first-order rate constant of hydrolysis of this tetrapeptide is found to be 3×10^{-9} s^{-1} and corresponds to a half-life of approximately 7 years.

Methods

To measure the slow rate at which amides are hydrolyzed in neutral water, it was necessary to devise a method for detecting and measuring the extremely low concentrations of hydrolysis products which would accumulate during the hydrolysis experiments. We expected such products to be formed over the course of hours to days in the parts per million to parts per billion range. To detect these products, radioassay was chosen because of its high sensitivity and convenient quantification by liquid scintillation counting (LSC).

Our plan was to covalently attach a radiolabeled peptide to an insoluble polymer and to detect amide hydrolysis by monitoring the escape of the radiolabel into the bulk solution. Thus, to a polymer support in the form of small beads (P2) we attach a peptide radiolabeled with ^{14}C (*) at the α-carbon of the C-terminal residue (Figure 1). The support is chosen and the peptide attached such that all bonds linking the radiolabel to the polymer backbone are either amide bonds or carbon–carbon bonds. Since the polymer used is both insoluble and stable in water, simple filtration removes any polymer-bound label from radiolabeled hydrolysis products. Scintillation counting of the filtrate thus measures the extent to which the radiolabel has been released into solution and thereby the rate at which peptide bonds are cleaved. There are of course other mechanisms by which radiolabel could escape from the polymer beads into solution, and a number of controls will be necessary to distinguish them from the amide hydrolysis we wish to detect.

For the polymer support we chose the polyacrylamide resin developed by Sheppard and co-workers for oligopeptide and oligonucleotide synthesis.[3] This resin consists of an all-carbon backbone with side chains containing only C–C and amide linkages. It is commercially available and swells in aqueous solvents,[4] and its reactive functionality is a methyl ester (loading ca. 0.3 mmol/g^{-1} of dry resin). To activate the resin for attachment of a peptide, the ester would be converted to an acyl azide via the corresponding hydrazide.[5] The bound peptide used for the hydrolysis experiments was chosen as P_2-(L)Phe(L)Phe(L)PheGlyOH (**4**). This amino acid sequence is of no intrinsic significance but was selected because of its affinity for certain PheGly binding sites which are under development here.

As shown in Scheme I, we prepared the radiolabeled tetrapeptide resin **4** by attaching purified triphenylalanine (Phe_3) to the activated Sheppard polyacrylamide resin and coupling labeled glycine as the last step to minimize the handling of hot material. We did not synthesize the peptide directly on the polyacrylamide resin because of the problems associated with N→C peptide coupling.[6] Instead the tripeptide (L)Phe_3 was synthesized on polystyrene (P_1) by using standard Merrifield methodology (BOC protection and DCC coupling), removed from the support with methanol and triethylamine as the CBZ-protected methyl ester (**1**), and purified by chromatography on silica gel. Treatment with hydrazine followed by $(tBuOCO)_2O$ gave a BOC-protected hydrazide precursor which was deprotected at the N-terminal position to the free amine **2** with hydrogenation using palladium on barium sulfate (81% yield overall).[7] Although the hydrazide could have been prepared directly by detaching the tripeptide from the Merrifield resin with hydrazine,[8] the longer route via the methyl ester was chosen because it facilitates purification and handling of the detached product.

The Sheppard resin was activated for coupling wtih hydrazine/DMF (1:1, 4 h) and then butyl nitrite in DMF (−5 °C, 30

(2) (a) Hershfield, R.; Bender, M. L. *J. Am. Chem. Soc.* **1972**, *94*, 1376. (b) Kunitake, T.; Okahata, Y.; Sakamoto, T. *J. Am. Chem. Soc.* **1976**, *98*, 7799. (c) Murakami, Y.; Aoyama, Y.; Kida, M.; Nakano, A. *Bull. Chem. Soc. Jpn.* **1977**, *50*, 3365. (d) Lehn, J.-M.; Sirlin, C. *J. Chem. Soc., Chem. Commun.* **1978**, *949*. (e) Breslow, R.; Trainor, G.; Ueno, A. *J. Am. Chem. Soc.* **1983**, *105*, 2739. (f) Cram, D. J.; Lam, P. Y.; Ho, S. P. *J. Am. Chem. Soc.* **1986**, *108*, 839. (g) Menger, F. M.; Ladika, M. *J. Am. Chem. Soc.* **1987**, *109*, 3145. (h) D'Souza, V. T.; Bender, M. L. *Acc. Chem. Res.* **1987**, *20*, 146 and references therein.

(3) (a) Atherton, E.; Clive, D. L. J.; Sheppard, R. C. *J. Am. Chem. Soc.* **1975**, *97*, 6584. (b) Arshady, R.; Atherton, E.; Clive, D. L. J.; Sheppard, R. C. *J. Chem. Soc., Perkin Trans. 1*, **1981**, 529.

(4) The Merrifield polystyrene resin cannot be used in aqueous solvents; see: Wieland, T.; Birr, C.; Flor, F. *Justus Liebigs Ann. Chem.* **1969**, *727*, 130.

(5) Meienhofer, J. In *The Peptides*; Gross, E., Meienhofer, J., Eds.; Acadamic: New York, 1979; Vol. 1, pp 202–205.

(6) Barany, G.; Merrifield, R. B. In *The Peptides*; Gross, E.; Meienhofer, J., Eds.; Academic: New York, 1980; Vol. 2, pp 3–254.

(7) Felix, A. M.; Merrifield, R. B. *J. Am. Chem. Soc.* **1970**, *92*, 1385.

(8) Ohno, M.; Anfinson, C. B. *J. Am. Chem. Soc.* **1967**, *89*, 5994.

min) to convert the resin ester functionalities into acyl azides. Coupling of **2** to the activated resin in DMF/$NaHCO_3$ at −5 °C for 24 h gave **3**. The course of the reaction was monitored by following the disappearance of the acyl azide stretching band at 2140 cm^{-1} in the infrared spectrum (IR) of the resin. The polyacrylamide resin is transparent in the 1720–2800-cm^{-1} range and many of its derivatives are thus readily analyzed by IR (KBr pellet). Interestingly, the bound acyl azide appeared rather stable in the absence of peptidic amine. Thus a sample of the acyl azide resin underwent only partial decomposition (ca. 50%) to isocyanate and other products during vacuum drying for 10 h at room temperature.

The resin-bound tripeptide **3** (2.00 g) was deprotected with HCl in acetic acid and oxidized to an acyl azide with butyl nitrite. Next, 0.02 mmol (1 mCi, 2.2×10^9 cpm) of [2-^{14}C]glycine was added and allowed to couple over a 36-h period at −5 °C in basic DMF/water. The reaction could be monitored by following both the disappearance of labeled glycine from solution (LSC) and the loss of the acyl azide stretching band from the IR spectrum of the resin. During the coupling, approximately 30% of the radioactivity was incorporated into the resin, leaving 1.5×10^9 cpm of radiolabel in the bulk solution. When incorporation of radiolabel ceased, excess cold glycine was added to cap any remaining reactive functionalities.

Finally, the resin was washed extensively, first with DMF and then with portions of 0.5 M saline as described in the Experimental Section. Initially, the rate at which radioactivity bled from the resin matrix was high. During the week-long series of washings, the amount of radiolabel which could be extracted from the resin decreased with each washing until it eventually stabilized near 200 cpm/100 mg of resin per hour. At this point, no further decrease in the rate of radiolabel release could be achieved by any washing procedure we could devise.

The specific activity of the washed resin was determined to be 3.0×10^7 cpm/100 mg by LSC of a suspension of the resin beads. This value was confirmed by 1 N NaOH hydrolysis (25 °C for 348 h) which liberated 3.5 (±0.5) $\times 10^7$ cpm/100 mg of resin. The radiolabeled PhePhePheGly resin **4** itself was not analyzed further. However, cold **4** was prepared as described above but with unlabeled glycine and was subjected to amino acid analysis which gave an average of 0.618 μmol of phenylalanine and 0.215 μmol of glycine/mg of resin.[9] Thus, the ratio of phenylalanine to glycine of 2.87:1 is close to that expected for Phe_3Gly, and the loading of peptide on the resin corresponds to functionalization of approximately two-thirds of the methyl ester sites on the original polyacrylamide resin.

Results and Discussion

Having prepared the washed resin as described, we measured the rate of radiolabel release in distilled, deionized water. As at the end of our washing sequence, the rate of radiolabel buildup in the pure-water liquid phase was found to be 200 cpm/100 mg of resin per hour. The process which we monitored thus has a pseudo-first-order rate constant of 3×10^{-9} s^{-1}. We believe the process is amide bond hydrolysis for the reasons which follow.

First, we measured the rate of radiolabel release over a wide range of pH's at 25 °C.[10] The results are graphed in Figure 2 and show that in the pH ranges 0–4 and 10–14, the rate of label release changes by approximately 1 order of magnitude per pH unit. This observation is consistent with a bond-cleavage process having [H^+] or [OH^-] in the rate-determining step. Between pH 4 and 10, however, the rate of label release varied by less than 1 order of magnitude. One explanation of this relative constancy is that the rate profile in the more neutral range reflects the superposition of three different mechanisms for amide bond hydrolysis (acid-, base-, and water-catalyzed). Another explanation is that the radiolabel release we see is an artifact of our experimental procedure and that the even slower rate of amide hydrolysis is being obscured by signals from other sources. For example, bound or unbound impurities present at levels below 1% of that of the bound peptide could easily give rise to a signal which represents release of 0.001% of the total label attached to the resin.

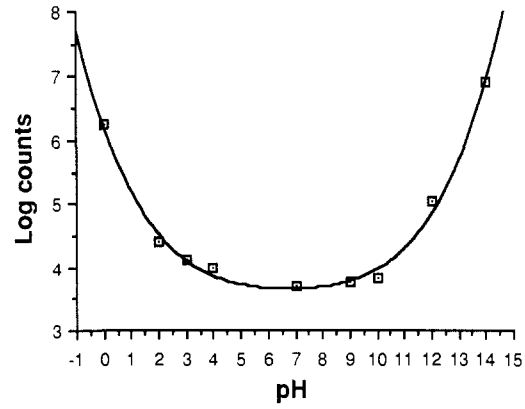


Figure 2. Release of radiolabel from resin **4** vs pH.

A series of experiments were therefore conducted to distinguish between amide bond hydrolysis and the folllowing processes: (1) mechanical breakdown of the resin and detection of the radioactive fragments, (2) incomplete washing of unbound radiolabeled glycine and detection of slow bleed from a reservoir of unbound material, and, (3) hydrolysis of an undefined but relatively labile chemical bond (e.g. an anhydride) which covalently binds the label to the resin.

Possibility 1. One explanation of a small, constant bleed of radiolabeled material from the polymer is that the polymer beads undergo simple mechanical degradation during agitation. The resulting small fragments might be able to pass through our filter and into the bulk solution where they would be detected by their radioactivity. Structural analysis of the cleaved fragments was therefore used to determine whether such degradation could account for the bulk of the LSC signal.

As shown in Figure 1, indiscriminant amide hydrolysis would result in the release of radiolabeled Gly, PheGly, PhePheGly, and PhePhePheGly. Resin degradation on the other hand would lead to the release of labeled peptide still attached to large fragments of polymer. To distinguish between these alternatives, the composition of the hydrolysis product was analyzed by HPLC. Since there is too little released material from the hydrolysis in neutral water at 25 °C for direct analysis (ca. 4 pmol/100 mg of resin per hour), we synthesized cold PheGly, PhePheGly, and PhePhePheGly and found reverse-phase HPLC conditions (Pharmacia PHR 5/5 C18, linear gradient, 0.7 mL/min, H_2O/0.1% TFA (v/v) to MeOH/0.1% TFA (v/v) over 40 min) which separated the three peptidic fragments and glycine. These cold materials were mixed with the radioactive concentrate from resin hydrolysis at pH 7. The resulting mixture was applied to the HPLC column and the eluent corresponding to each peak in the HPLC trace was collected and counted. We found that more than 95% of the radioactivity released by neutral-water hydrolysis appeared at the position of the glycine peak. The radioactivity also moved with authentic glycine with use of a number of different HPLC eluents. Similar results were obtained from analysis of the products of the pH 3 hydrolysis. Thus, both neutral and acidic conditions preferentially liberate glycine and mechanical breakdown of the polymer matrix may therefore be excluded as the main source of radiolabel release from the polymer beads. Furthermore, these results are not a function of the agitation method used during the washing sequence since neither vigorous shaking nor sonication increased the rate of radiolabel release into the filtrate to a measurable extent.

Possibility 2. The most trivial mechanism by which glycine could be slowly released from the polymer beads is by slow diffusion from some relatively inaccessible region of the polymer matrix where the hot glycine used in the synthesis might have become entrapped. Indeed, if the beads are dried and then res-

(9) We wish to thank Dr. Arthur M. Felix of Hoffmann-La Roche in Nutley, NJ, for the amino acid analyses.

(10) A universal buffer was used between pH 2 and 12; see: Davies, M. T. *Analyst* **1959**, *84*, 248. 1 N NaOH and 1 N HCl were used at pH 14 and 0, respectively.

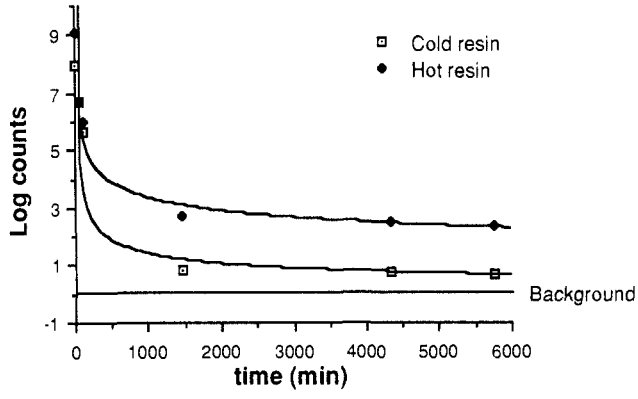


Figure 3. Release of radiolabel vs washing of polymer **4**.

wollen with solvent before the entire washing procedure is complete, it becomes difficult to reduce the radiolabeled glycine signal to the minimal 200 cpm level discussed above. To test for the development of reservoirs of entrapped radiolabeled glycine as the source of our glycine signal, we conducted the experiments below.

First we coupled CBZ(L)Phe_3N_3 to [2-^{14}C]glycine in solution and purified the hot tetrapeptide by reverse-phase HPLC and gel filtration (Sephedex LH20, 9 mm/1 m, 0.8 mL/min, MeOH). Next, the purified, radiolabeled tetrapeptide was coupled to the azide-activated Sheppard polyacrylamide resin to provide **4**. When this sample of **4** was washed to a constant rate of label release, the neutral-water signal again amounted to approximately 200 cpm/100 mg of resin per hour. Again, the signal was predominantly due to glycine even though radiolabeled glycine itself had not been coupled on the resin. While this experiment would seem to rule out incomplete washing as the source of the glycine signal, such a conclusion is unwarranted since hot glycine is slowly cleaved from the tetrapeptide during HPLC purification (0.01% TFA/CH_3OH) and coupling to the P_2 resin (aqueous $NaHCO_3$, pH 9). By the time the purification and coupling of the tetrapeptide are complete, approximately 1% of the total radioactivity in the bulk solution is in the form of free glycine. This 1% amounts to 10^5 cpm and it is therefore formally possible that selective incorporation of the free glycine into the polymer could give rise to the observed 200 cpm signal. It is important to note, however, that **4** prepared by the two independent methods gave the same 200 cpm bleed/h in pure water in spite of the 1000-fold difference in the amount of free labeled glycine to which the resin had been exposed.

The second experiment has fewer ambiguities and was designed to test our ability to wash unbound glycine from the resin after the coupling reaction. To this end, azide-activated (L)Phe_3 resin was prepared from **3** and coupled with *cold* glycine as in the previously described synthesis of radiolabeled **4**. After 36 h at −5 °C the coupling was complete according to IR analysis of the resin. Next, 10^8 cpm of radiolabeled glycine was added to the reaction mixture and the bead suspension was agitated for an additional 36 h, allowing the glycine to equilibrate between bulk solution and the resin matrix. If slow diffusion of trapped, labeled glycine from reservoirs within the polymer matrix accounted for our basal signal, then it should not be possible to wash this new, covalently cold resin to a level of glycine bleed below the 200 cpm/100 mg of resin per hour. The results of LSC monitoring of the washing experiments are shown in Figure 3.

Whereas the original resin **4** could be washed only to 200 cpm/100 mg resin per hour, the new cold resin could be washed down to approximately 5 cpm/100 mg of resin per hour in the same length of time using an identical washing protocol. Thus, our washing procedure appears to eliminate unbound glycine effectively. The 200 cpm signal we observe with hot resin **4** must therefore result from release of hot glycine which is chemically bound to the resin.

Possibility 3. Since the above experiments establish that we are cleaving bound glycine from the resin, the remaining question concerns the nature of the scissile bond to glycine. While most of the glycine is bound to the resin as the C-terminal residue of R_2-PhePhePheGly, our hydrolyses in neutral water remove only minuscule fractions (<0.01%) of the total radioactivity on the resin. Undefined but reactive functionalities binding as little as 0.1% of the total radioactivity could easily give rise to slow release of the radiolabeled glycine we detect. It is difficult to rule out this alternative explanation of our results unambiguously, but it is possible to test for reasonable manifestations.

To this end, samples of resin **4** were treated with 1 N HCl and 1 N NaOH to remove a substantial amount of the radioactivity from the resin. By scintillation counting the bulk acid and base solutions, it was found that 7% and 51%, respectively, of the total radiolabeled glycine had been detected from the two samples of resin. As noted previously, complete hydrolysis established the total amount of radioactive glycine to be approximately 3.5×10^7 cpm/100 mg of resin. The acid- and base-hydrolyzed resin samples (100 mg) were then washed and the minimal bleed rates were found to be 162 and 100 cpm/h, respectively, in neutral water at 25 °C. These rates compare well to the 186 and 98 cpm/h signals which would be expected after removal of 7% and 51% of the labeled glycine from the original resin. This result would be compatible with cleavage of nonpeptidically bound glycine only if the scissile bond's rate of cleavage in both strong acid and strong base were close to that of the terminal glycine of P_2-PhePhePheGly *and* the bond was much more labile than an amide linkage at neutral pH. We know of no functionality which has these characteristics. The most likely explanation for our results is amide hydrolysis.

Conclusion

In the paragraphs above, we described a solid-phase radioassay which was designed to detect amide bond hydrolysis by measurement of radiolabel release from the solid support. We used LSC to detect the expected signal and showed that physical degradation of the resin is unlikely to confuse the assay since the bulk of the radioactivity released was in the form of glycine. We prepared the resin **4** by a route not involving exposure of the resin to large quantities of hot glycine and verified the efficiency of our washing protocol as evidence that chemically bound glycine was the source of the label release. Finally, we presented evidence which strongly suggested that P2-PhePhePheGly is the precursor of the glycine signal we see at neutral pH in 25 °C water. We conclude that the 200 cpm release of labeled glycine from 100 mg of resin **4** per hour in pure water at room temperature is due to hydrolysis of the terminal PheGly peptide linkage.

The fact that most of the released radioactivity was in the form of glycine is noteworthy. It may reflect the relative solvent accessibility of the hydrophilic, C-terminal residue, or it may be the result of hydrolytic assistance by the proximate carboxylate.[11a] Peptide bonds to aspartic acid, for example, are known to be preferentially cleaved in dilute acid.[11b-d] In any event, the other possible cleavage sites appear less favorable by a factor of 10^2 or more according to HPLC analysis of our hydrolysates which showed only traces of PheGly and PhePheGly. Phe_3Gly could not be detected but its absence could reflect an unfavorable partitioning of the hydrophobic tetrapeptide between the organic polymer and the aqueous bulk phase.

One final point we wish to establish is that the hydrolysis we detect is due to water itself and not to the presence of catalytic impurities. Thus, autoclaved and unautoclaved buffers gave the same results, as did buffers containing sodium azide. Similar rates of glycine release were also found with P_2-(D)Phe(D)Phe(D)-PheGlyOH.[12] Taken together, these results suggest that biological

(11) (a) Kirby, A. J. *Advances in Physical Organic Chemistry*; Gold, V., Bethell, D., Eds.; Academic: 1980; Vol. 17, pp 183–278. (b) Marcus, F. *Int. J. Pept. Protein Res.* **1985**, *25*, 542. (c) Garett, E. R. *J. Am. Chem. Soc.* **1960**, *82*, 711. (d) Piszkiewicz, D.; Landon, M.; Smith, E. L. *Biochem. Biophys. Res. Commun.* **1970**, *40*, 1173.

(12) We cannot rule out small amounts of phenylalanine racemization during synthesis.

(13) Stewart, J. M.; Young, J. D. *Solid Phase Peptide Synthesis*; Pierce Chemical Co.: Rockford, IL, 1984; pp 70–71.

catalysts do not account for the amide hydrolysis we observe. The possibility that trace metals in the water might catalyze hydrolysis also seems unlikely since distilled, deionized water was used in all experiments. Moreover, we directly tested the effects of common metal ions (10 mM Co^{2+}, 10 mM Cu^{2+}, 10 mM Zn^{2+}, 10 mM Ni^{2+}) on the rates and, after correcting for differences in pH, found no measurable acceleration (±10%). Changes in ionic strength (0–0.5 M NaCl) had no affect, nor did any of the common organic moieties: acetate (pH 7.5, 10 mM), imidazole (pH 7, 2.5–250 mM), methanol (pH 7.5, 10 mM), or phosphate (pH 7, 5–500 mM).

The work described here strongly suggests that the rate of simple amide hydrolysis in water can be detected and measured with use of a polymer-bound radiolabeled substrate. The main advantage of the resin-based assay described here is that it permits ready separation of starting materials from radiolabeled hydrolysis products. Thus the starting material background signal is minimal and extremely low levels of hydrolysis product can be detected. The sensitivity of our assay is limited only by the extent of radiolabel incorporation which can be achieved (or afforded). Assuming that amide cleavage from the water-swollen polyacrylamide resin is closely related to hydrolysis in aqueous solution, the assay provides a sensitive, quantitative method with which to evaluate new molecules for real peptidase activity.[19]

Experimental Section

Synthesis of CBZPhePhePheOMe (1). The cesium salt of BOC-L-Phe (796 mg, 3.0 mmol) was attached to the chloromethylated polystyrene resin (Bio-Rad, Bio-Beads, S-X1, 200–400 mesh, 1.25 mequiv g^{-1}, 3 g) by standard methods.[13] After shaking of the mixture for 24 h (DMF, 60 °C), the solvent was removed by filtration through a sintered-glass funnel and the resin was washed with the following solvents: H_2O/DMF (1:9, 100 mL), DMF (100 mL), EtOH (10 mL), and CH_2Cl_2 (200 mL). Resin-bound BOC-L-Phe (3.392 g) was obtained after it was dried under high vaccum (loading ca. 0.75 mequiv g^{-1} by using the Gisen test).[14]

The derivatized resin (3.392 g) was then swollen in CH_2Cl_2 and washed with the following solvents to deprotect the amino acid: CH_2Cl_2 (80 mL, 5 × 1 min), 45% TFA/CH_2Cl_2 (80 mL, 3 × 1 min), 45% TFA/CH_2Cl_2 (80 mL, 1 × 2 h), CH_2Cl_2 (80 mL, 5 × 13 min), 5% DIEA/CH_2Cl_2 (80 mL, 3 × 5 min), and CH_2Cl_2 (80 mL, 5 × 1 min). Three equivalents of BOC-L-Phe (2.022 g, 7.63 mmol) was then added to the shaker containing the resin in CH_2Cl_2 (50 mL). After shaking of the mixture for 5 min, 0.3 equiv of DCC was added (1.572 g, 7.63 mmol) in CH_2Cl_2 (10 mL) and the reaction mixture shaken for 2 h. The resin was filtered and then washed with the following solvents: CH_2Cl_2 (80 mL, 2 × 1 min), MeOH (80 mL, 1 × 1 min), and CH_2Cl_2 (2 × 1 min). With the same deprotection/coupling sequence, CBZ-(L)Phe (2.282 g, 7.63 mmol) was then coupled to the resin to produce the protected tripeptide. Cleavage of the tripeptide from the resin was accomplished by transesterification in MeOH/Et_3N (4:1, room temperature, 5 days). After column chromatography on silica gel (5% MeOH/$CHCl_3$), 1.216 g of **1** (89%) was obtained as a white solid: ^1H NMR ($CDCl_3$, 270 MHz) δ 7.38–6.90 (m, 20 H, Ar *H*), 6.34 (d, J = 7 Hz, 1 H, NH), 6.14 (d, J = 7 Hz, 1 H, NH), 5.10 (d, J = 7 Hz, 1 H, NH), 5.04 (d, J = 13 Hz, 1 H, OCH_AH_BPh), 4.99 (d, J = 13 Hz, 1 H, OCH_AH_BPh), 4.70 (q, J = 7 Hz, 1 H, C*H*NH), 4.52 (q, J = 7 Hz, C*H*NH), 4.34 (q, J = 7 Hz, 1 H, C*H*NH), 3.66 (s, 3 H, OCH_3), 3.12 (m, 6 H (CH_2Ph); IR ($CHCl_3$) 3300, 3060, 1738, 1694, 1639 cm^{-1}; mass spectrum (CI, NH_3), m/e 608 (M + 1), 500.

Conversion of CBZPhePhePheOMe (1) to H_2NPhePhePheNHNHBOC (2). To a dry 25-mL flask was added 0.313 mmol of the tripeptide methyl ester **1** (190 mg) in 6 mL of anhydrous DMF/hydrazine (1:1) (Aldrich). After stirring for 30 min at room temperature the reaction was complete, as judged by TLC (10% MeOH/$CHCl_3$), and the solvent mixture was removed under reduced pressure. The crude hydrazide, a white solid, was used directly in the next reaction.

To a dry 25-mL flask was added 0.313 mmol of the tripeptide hydrazide (190 mg) in 5 mL of anhydrous DMF. After the hydrazide had dissolved completely (10 min), 2 equiv of BOC pyrocarbonate (Fluka, 121 mg, 0.626 mmol) and 1 equiv of freshly distilled DIEA (54 μL, 0.313 mmol) were added. After the reaction was complete, as judged by TLC (10% MeOH/$CHCl_3$), the solvent was removed under reduced pressure. Chromatography (5% MeOH/$CHCl_3$) on silica gel afforded 205 mg of the BOC hydrazide (0.290 mmol, 93%) over the two steps.

Removal of the carbobenzyloxy protecting group was accomplished by following the procedure of Merrifield.[7] A 25-mL flask was charged with 5 mg (0.0145 mmol, 5 mol %) of Pd/$BaSO_4$ and then evacuated and filled several times with hydrogen. To this flask was added a solution of 205 mg (0.290 mmol) of the protected tripeptide BOC hydrazide in 6 mL of MeOH and the reaction was stirred at room temperature. After the reaction was complete, as judged by TLC (10% MeOH/$CHCl_3$), the solution was filtered through a plug of Celite to remove the catalyst. Chromatography on silica gel (10% MeOH/$CHCl_3$) gave 143 mg of the desired compound **2** (0.249 mmol, 89%) as a white solid: ^1H NMR (CD_3OD, 270 MHz) δ 7.44–7.15 (m, 15 H, Ar H's), 4.84 (m, 2 H, C*H*NH), 3.95 (dd, J = 4 and 9 Hz, 1 H, C*H*NH), 3.36–2.79 (m, 6 H, CH_2Ph), 1.56 (s, 9 H, C_4H_9); IR (neat) 3275, 3030, 1675 cm^{-1}; mass spectrum (CI, methane), m/e 574 (M + 1) 474.

Preparation of the Polyacrylamide Resin Derivatized with the Tripeptide BOC Hydrazide 3. Polyamide peptide resin II (Chemical Dynamics, loading 0.3 mequiv/g, 600 mg) was added to a silanized jacketed shaker vessel[13] containing 20 mL of anhydrous DMF/hydrazine (1:1) and shaken at room temperature.[15] After 4 h, IR examination of the resin showed no ester stretch (1745 cm^{-1}, KBr pellet), indicating that the reaction was complete. The solvent was removed by filtration through a sintered-glass funnel and the resin washed with DMF (20 mL, 5 × 1 min).

The jacketed shaker containing the acyl hydrazide in DMF (20 mL) was cooled to −5 °C, and 2 N HCl (2 mL, 4 mmol, 20 equiv) followed by 236 μL of butyl nitrite (2 mmol, 10 equiv) was added.[16] After shaking of the mixture for 30 min at −5 °C, the solvent was removed by filtration as before and the resin washed with DMF (precooled to 0 °C, 20 mL, 3 × 1 min). IR examination of a dried portion of the resin showed the presence of an azide (2140 cm^{-1}, KBr pellet).

Tripeptide **2** (340 mg, 0.593 mmol, 3.3 equiv) was added to the jacketed shaker containing 10% DIEA/DMF (20 mL) and the resin-bound acyl azide at −5 °C and shaken for 24 h at −5 °C.[7,17] The shaker was then warmed to room temperature. After 12 h the solvent was removed by filtration and the resin washed with DMF (20 mL) and then CH_2Cl_2 (20 mL, 3 × 3 min). IR examination of a sample of resin showed no acyl azide. Unreacted tripeptide **2** (258 mg, 0.450 mmol) was recovered from the filtrate, leaving 0.143 mmol of peptide bound to the resin. From this, we calculate that 79% of the available sites on the resin (0.18 mequiv/600 mg) were derivatized.

Preparation of the Polyamide Resin Derivatized with the Radiolabeled Tetrapeptide 4. The polyamide resin containing the tripeptide BOC hydrazide **3** was washed in a sintered-glass shaker vessel with 20 mL of concentrated HCl/glacial HOAc[3] (1:9, 3 × 5 min) and then shaken with 20 mL of HCl/HOAc (1:9, 3 h) to remove the protecting group. After deprotection, the solvent was removed by suction through the sinter and the resin was washed with *tert*-amyl alcohol (20 mL, 30 min) and then with DMF (20 mL, 5 × 1 min).

The jacketed shaker vessel containing the resin-bound tripeptide hydrazide in DMF (20 mL) was cooled to −5 °C. HCl (2N, 2 mL) followed by 236 μL of *n*-butyl nitrite (2 mmol, ca. 10 equiv) was added and the mixture was shaken for 30 min. The solvent was removed by suction and the resin washed in DMF (0 °C, 20 mL, 1 × 5 min) and then 10% DIEA/DMF (0 °C, 20 mL, 3 × 5 min). A sample of resin was removed and dried under high vacuum. IR examination of the resin showed the presence of an azide (2140 cm^{-1}, KBr pellet).

To the jacketed shaker containing the acyl azide was added a precooled solution of 5% DIEA/DMF (−5 °C, 7 mL). To this solution was added 50 μCi of [2-^{14}C]glycine (New England Nuclear, 40–60 mCi/mmol) as an aqueous solution (14 mL). (The radiolabeled glycine comes in a 0.1 N HCl solution and was first neutralized with $NaHCO_3$.) The reaction vessel was shaken at −5 °C and the progress of the reaction followed by monitoring the decrease in radioactivity in free solution over time. After 36 h at 0 °C, the reaction appeared to be complete and 135 mg of cold glycine was added (1.8 mmol, 10 equiv) in a minimum volume of water to cap any unreacted acyl azide. After 12 h the shaker was warmed to room temperature. The polyamide resin containing the terminally radiolabeled tetrapeptide 4 was then washed to remove unreacted [2-^{14}C]glycine.

Washing of the Polyamide Resin Derivatized with the Terminally Radiolabeled Tetrapeptide 4. After coupling the [2-^{14}C]glycine to the polymer-bound tripeptide, the resin **4** (2.00 g) was washed in the sin-

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(19) We gratefully acknowledge NIH Grant GM 37225 and NSF Grant CHE86 05891 for supporting this work.

tered-glass shaker with a NaCl solution (0.5 M, 50 °C, 60 mL, 10 × 5 min), DMF (room temperature 60 mL, 10 × 5 min), and H_2O (room temperature, 60 mL, 10 × 5 min). Following the three-step washing sequence, the resin was shaken in NaCl solution (0.5 M, room temperature, 60 mL) for 1 h and an aliquot was filtered and counted to monitor the rate of bleed. The washing procedure was repeated (5–10 cycles) until the bleed rate fell below 1000 cpm/100 mg per hour (this value is based on a specific activity of 10^7 cpm/100 mg). Finally, the resin was rinsed with CH_2Cl_2 (60 mL, 3 × 5 min) and the solvent is removed under high vacuum overnight. Note that it is important that the bulk of the unattached glycine be removed by washing before the resin is dried for storage. Otherwise, it takes considerably longer for the rate of bleed to reach a constant (base-line) level upon reswelling the dried beads.

Synthesis and Washing of the Polyamide Resin Derivatized with Unlabeled Tetrapeptide. The cold tetrapeptide was synthesized on the resin by using the same procedure as for the radiolabeled tetrapeptide, except that cold glycine was used in place of the [2-^{14}C]glycine (−5 °C, 36 h). Before warming of the mixture to room temperature, 50 μCi of labeled glycine was added to the resin derivatized with the cold tetrapeptide. The resin mixture was warmed to room temperature and shaken for 24 h. The resin was then washed and the radioactivity monitored as described for the radiolabeled tetrapeptide.

General Procedure for Measuring Rates of Hydrolysis. A sample of polyamide resin derivatized with labeled tetrapeptide **4** (100 mg) was put in a 1-dram vial. The appropriate buffer (4 mL) was added and the vial was shaken for 24 h on a Milligen Model 504 shaker. The vial was centrifuged for 1 min (1000 rpm, IEC Clinical Centifuge), and two 1-mL aliquoits were carefully withdrawn from the top and filtered through a cotton plug into glass scintillation vials. Hionic-fluor (9 mL, Packard) was added, the vials were shaken by hand, and the radioactivity was counted (Packard Tri Carb Model 1500 scintillation counter).

Fresh buffer (2 mL) was added to the 1-dram vial containing the resin. After shaking of the mixture for 24 h, two 1-mL samples were withdrawn and counted as before. This procedure was repeated 12 more times (14 total), and the number of new counts produced every 24 h was calculated by using the following formula:[18]

$$2 \times [2\ (\text{counts}/1\ \text{mL of sample after 24 h}) - (\text{counts}/1\ \text{mL of sample as start})] = \text{total new counts produced in 24 h}$$

The data for the last 72 h was averaged and the mean value was used to calculate a rate constant by using the following formulae:

$$k = V/[S]$$

$$V = [\text{av number of new counts}/24\ \text{h (for 100 mg of resin)}] \times [24\ \text{h}/86400\ \text{s}]\ [1\ \mu\text{Ci}/2.2 \times 10^6\ \text{counts}] \times [1\ \mu\text{mol}/50\ \mu\text{Ci}]$$

$$[S] = [3 \times 10^7\ \text{counts (for 100 mg of resin)}] \times [1\mu\text{mol}/50\ \mu\text{Ci}] \times [1\ \mu\text{Ci}/2.2 \times 10^6\ \text{counts}]$$

Communications to the Editor

CO_2-Laser Desorption and Multiphoton Ionization of Tris(2,2′-bipyridyl)ruthenium

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Elucidation of the spectroscopy of metal coordination compounds is an important ingredient in arriving at an understanding of their photophysical and photochemical properties. However, in solution and the condensed phase, most metal complexes and organometallic compounds display electronic spectra that are environmentally broadened and are therefore relatively uninformative, and the low volatility of these species usually precludes gas-phase studies.[4,5] Ideally, it would be desirable to measure spectra of metal coordination compounds under molecular beam conditions where the cooling and rarefaction afforded by expansion would assist in reducing thermal congestion in the electronic spectrum.[6] The spectroscopy of ruthenium polypyridyl complexes is of particular interest, because of their relevance as photosensitizers for solar energy conversion schemes.[7] However, to our knowledge there have been no successful attempts to introduce such molecules into the gas phase for measurement of their spectroscopy.

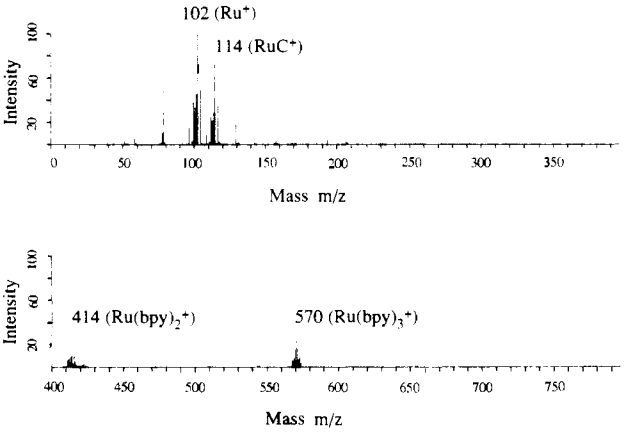

Figure 1. Resonance enhanced multiphoton ionization time-of-flight mass spectrum following CO_2-laser desorption of $[Ru(bpy)_3](CH_3COO)_2$.

We have succeeded in producing a seeded molecular beam expansion of the tris(2,2′-bipyridyl)ruthenium moiety by using a pulsed CO_2-laser desorption technique[8-10] that has been employed successfully to introduce many other involatile species into molecular beam expansions for analytical[11] purposes.

Details of the CO_2-laser desorption and multiphoton ionization technique have been described elsewhere.[8-10] In the present ap-

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