of leucine per gram of resin according to amino acid analysis after hydrolysis in a mixture of dioxane–HCl.[18]

Stability of N-Bpoc Amino Protecting Group and the *t*-Alkyloxycarbonylhydrazide Anchoring Bond. A sample of Bpoc-Leu-resin (53 mg) was suspended in 1 ml of CH_2Cl_2 for a few minutes to allow the resin to swell. An equal volume of 1% TFA in the same solvent was then added and 0.1-ml samples were withdrawn at different time intervals. The resin particles were removed by filtration and washed several times with small volumes of CH_2Cl_2. The combined filtrate and washings were evaporated to dryness, dissolved in 24 ml of ethanol, and their absorbance at 254 mμ was measured to determine the amount of 2-(*p*-biphenyl)isopropyl alcohol present[10,11] with the results shown in Figure 2a. Trifluoroacetic acid was omitted from the control experiments. It can be seen that the removal of the Bpoc group was complete in about 10 min.

To see how stable the anchoring bond was under these conditions, several resin samples (VI) were prepared and suspended in 0.5% TFA in CH_2Cl_2 (30 mg/ml) in tightly capped small vials. At different times, one of the samples was taken and filtered and washed as described above. The liberated leucine hydrazide was then hydrolyzed with 6 *N* HCl at 105° for 24 hr. The leucine content of these samples is inversely related to the stability of the anchoring bond. Results of such experiments showed that there was only about 6% loss of the anchoring bond in 10 hr (see Figure 2b) which corresponded to 40 cycles of Bpoc deprotection according to the synthetic scheme described in the next section.

Z-Phe-Val-Ala-Leu-HNNH$_2$ (VIII). Bpoc-Leu-HNNH-resin (VI) (550 mg, 0.159 mmol) was placed in a peptide reaction vessel on a shaker[19] and treated as follows with 15-ml portions of solvents: (1) wash three times with CH_2Cl_2, (2) wash once with 0.5% TFA in CH_2Cl_2, (3) shake 10 min with 0.5% TFA in CH_2Cl_2, (4) wash three times with CH_2Cl_2, (5) wash once with 10% DIEA[4] in CH_2Cl_2, (6) wash three times with CH_2Cl_2, (7) wash three times with EtOH, (8) wash three times with CH_2Cl_2, (9) shake 10 min with 10% DIEA in CH_2Cl_2, (10) wash three times with CH_2Cl_2, (11) soak 10 min with 210 mg (0.64 mmole) of Bpoc-L-Ala in 6 ml of CH_2Cl_2, then add 132 mg (0.64 mmol) of DCC in 6 ml of CH_2Cl_2 and shake 90 min, (12) wash three times with CH_2Cl_2, (13) wash three times with DMF, (14) wash three times with EtOH. The cycle was repeated with 228 mg (0.64 mmol) of Bpoc-L-Val in step 11, and again with 185 mg (0.64 mmole) of Z-L-Phe. In each cycle a fourfold excess of amino acid derivative was used. The protected peptide hydrazide resin (VII) thus obtained weighed 610 mg after drying. According to the amino acid analysis, this material contained 0.232 mmole of peptide per gram of resin and had an amino acid composition of $Ala_{0.97}$ $Val_{1.00}$ $Leu_{0.97}$ $Phe_{1.05}$. The yield of the peptide at this stage was 89% calculated from the leucine content of VI.

The protected peptide hydrazide was liberated from the solid support by shaking 500 mg (0.116 mmole) of VII with 12 ml of 50% TFA in CH_2Cl_2 (v/v) at room temperature for 30 min. The resin was removed by filtration and washed with a few milliliters of CH_2Cl_2. After removal of the solvent by evaporation under reduced pressure, the peptide hydrazide was obtained as a white powder. It was then crystallized from methanol by addition of ether to give 60 mg (0.101 mmol) of the product melting at 255–257°. It had an amino acid composition of $Ala_{0.99}$ $Val_{1.00}$ $Leu_{1.04}$-$Phe_{1.00}$.

Anal. Calcd for $C_{31}H_{44}N_6O_6$ (596.71): C, 62.30; H, 7.43; N, 14.08. Found: C, 62.21; H, 7.40; N, 14.10.

Acknowledgments. We wish to thank Miss D. M. Cohen for her technical assistance in the preparation of Bpoc-amino acid derivatives, Miss U. Birkenmaier for carrying out the amino acid analyses, and Mr. S. T. Bella for performing the microanalysis.

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(19) R. B. Merrifield and M. A. Corigliano, "Biochemical Preparations," Vol. 12, W. E. M. Lands, Ed., John Wiley & Sons, Inc., New York, N. Y., 1968, p 98.

The Pepsin-Catalyzed Hydrolysis of Bis-*p*-nitrophenyl Sulfite and Its Inhibition by Diphenyl Sulfite at pH 2

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Abstract: Bis-*p*-nitrophenyl sulfite, BNPS, is an excellent substrate for the proteolytic enzyme pepsin at pH 2. The enzyme-catalyzed hydrolysis of this sulfite proceeds approximately 10^3 times faster than that of other known sulfite esters. This rate acceleration is apparently due principally to the high value of k_{cat}, the catalytic rate constant, for the enzymatic hydrolysis of BNPS. The Michaelis constant for the pepsin-catalyzed hydrolysis of the nitro-substituted aromatic sulfite ester is not very different from that which is observed in the case of the unsubstituted ester diphenyl sulfite, DPS. We have studied the inhibition of the enzymatic hydrolysis of BNPS by added DPS. The inhibition constant, K_I, which we have measured in this way for DPS differs appreciably from the Michaelis constant we have found for the pepsin-catalyzed hydrolysis of DPS.

Recently, much evidence has been presented that the Michaelis constants, K_M, obtained from kinetic analyses of pepsin-catalyzed reactions are simple dissociation constants.[3-5] This would imply that in the reaction scheme represented by eq 1 which is likely to apply for pepsin-catalyzed hydrolyses,[5-8] k_2 is the rate-

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(3) W. T. Jackson, M. Schlamowitz, and A. Shaw, *Biochemistry*, 4, 1537 (1965).

(4) K. Inouye and J. S. Fruton, *J. Am. Chem. Soc.*, 89, 187 (1967).

(5) E. Zeffren and E. T. Kaiser, *Arch. Biochem. Biophys.*, 126, 965 (1968).

(6) E. Zeffren, Ph.D. Thesis, University of Chicago, 1967, p 99.

(7) T. P. Stein and D. Fahrney, *Chem. Commun.*, 555 (1968).

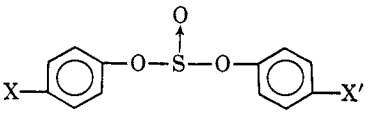
(8) The abbreviations used in this paper are: acetyl, Ac; diphenyl sulfite, DPS; bis-*p*-nitrophenyl sulfite, BNPS; dibromotyrosine, Di-BrTyr; phenylalanine, Phe. v_0 is the initial rate of an enzymatic reaction, and k_{cat} and K_M are the catalytic rate constant and Michaelis constant, respectively, for such a reaction. K_I^c is the inhibition constant calculated for acetonitrile if this substance is treated as a competitive inhibitor. All amino acid residues are of the L configuration unless otherwise specified. ES represents a Michaelis complex and ES′ is a covalent species.

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_1 + P_2\,(+P_3) \quad (1)^9$$

limiting step. However, all of these studies have dealt with peptide substrates. It is known that in the case of chymotrypsin, certain active ester substrates exhibit a rate-determining deacylation step,[10] in contrast to the observation of a rate-determining acylation step for various amide substrates.[11] It was thought that the same could be true for active ester substrates of pepsin (*i.e.*, $k_3 < k_2$) which would make the apparent Michaelis constant $K_M < K_s$ ($K_M = K_s[k_3/(k_2 + k_3)]$.

Also, in all cases except one,[5] the evidence for the identity of K_M to K_s, which has been postulated frequently for pepsin-catalyzed peptide hydrolyses, is indirectly inferred from the identity of K_M values found for L-L diastereomers of specific peptide substrates to the K_I values (inhibition constants) observed for the corresponding DL-diastereomers.[3,4,12]

Therefore, it seemed desirable to try to find a pepsin substrate which would hydrolyze sufficiently rapidly so that the inhibition of its hydrolysis by a pepsin ester substrate could be measured. The fact that the value of k_{cat}/K_M for the hydrolysis of bis-*p*-bromophenyl sulfite (**Ib**) is six times as large as that for diphenyl sulfite (DPS, Ia)[13] suggested to us that bis-*p*-nitrophenyl sulfite (BNPS, Ic) might be such a substrate. In addition, it was attractive to us that a *p*-nitrophenol chromophore would be generated during the hydrolysis of Ic making it possible to follow the enzymatic reaction spectrophotometrically at a wavelength (325 mμ) where the background absorption of pepsin and most sulfite esters is small.



Ia, X = X′ = H
b, X = X′ = Br
c, X = X′ = NO_2

We report herein studies on the pepsin-catalyzed hydrolysis of BNPS at pH 2.0 and on the use of this substrate in the determination of K_I values for pepsin inhibitors.

Experimental Section

Synthesis. BNPS was synthesized by the reaction of *p*-nitrophenol (2 equiv) and thionyl chloride (1 equiv) in the presence of pyridine (2 equiv) in benzene at 0°. The benzene layer was decanted and the residue washed with benzene. Evaporation of the solvent left crude BNPS. This was purified by recrystallization from chloroform–hexane at room temperature. At least four recrystallizations were necessary to give pure BNPS in very low yield, mp 91–92°.

Anal. Calcd for $C_{18}H_8N_2O_7S$: C, 44.44; H, 2.49. Found: C, 44.36; H, 2.50.

Materials. Pepsin, twice recrystallized and lyophilized, was purchased from Worthington Biochemical Corp. (Lot No. PM 8BE and PM-718). Enzyme solutions were prepared by dissolving a given amount of pepsin in 40.0 ml of buffer of the desired ionic strength and stirring the resultant mixture using a magnetic stirrer for 30–45 min. When pepsin concentrations $>10^{-5}$ *M* were desired, the solutions were then filtered through a Millipore filter. The pH values of the solutions were adjusted to 2.0, and the enzyme concentrations were determined spectrophotometrically at 278 mμ on a Cary 15 recording spectrophotometer using $\epsilon_{278} = 51{,}500$.[14] Enzyme solutions were stored at 4° for a maximum of 5 days. Successive assays of a given enzyme solution showed that after 5 days, pepsin activity had decreased no more than 2%.

DPS was a gift from Dr. K. W. Lo, and its synthesis is described elsewhere,[15] bp 144–145° (3.5–4 mm).

AcPhe was purchased from the Sigma Chemical Corp. (lot No. A305B-58) and was used without further purification.

Acetonitrile was freshly distilled from P_2O_5 before use, bp 80.1°.

Deionized water was obtained from a Continental mixed-bed ion exchange column (Continental Demineralization Service, Chicago, Ill.). For stopped-flow measurements, deionized water was degassed using an aspirator for 30 min to minimize subsequent bubble formation in the stopped flow instrument.

Stock solutions of both DPS and BNPS were prepared by dissolving a given weight of the appropriate ester in distilled acetonitrile. DPS solutions were stored in a refrigerator at 4° and were used within 1 week of their preparation. BNPS stock solutions were stored in a freezer at −30°, at which temperature they decomposed slowly. These solutions were freshly prepared every few days.

A stock solution of AcPhe was prepared by dissolving 0.5190 g of AcPhe in 2.9 ml of 1 *N* NaOH and diluting the solution to 5 ml with deionized water to give a 0.501 *M* stock solution which was stored at 4°.

Phosphate buffers were prepared by mixing 0.1 *M* stock solutions of NaH_2PO_4 and H_3PO_4. They were diluted to the proper ionic strength with H_2O when desired.

All pH measurements were made with a Radiometer pH meter, Type 4C, standardized before each determination with pH 2.0 standard buffer (Fisher Certified).

Kinetic Measurements. The hydrolysis of BNPS was studied on a Durrum-Gibson stopped-flow spectrophotometer (Durrum Instrument Corp., Palo Alto, Calif.). All runs were performed at pH 2.0 and 25.0 ± 0.1°, and under conditions of enzyme in excess over the substrate. The reaction was followed at 325 mμ. One syringe was filled with enzyme solution of the appropriate concentration in phosphate buffer, $\mu = 0.05$, pH 2.0, and allowed to equilibrate to 25.0°. The second syringe was filled with a solution prepared in the following manner: distilled, deionized, and degassed water, equilibrated to 25°, distilled acetonitrile, BNPS stock solution, and inhibitor (if any) were mixed in appropriate proportions to give the desired final inhibitor concentration and percentage of acetonitrile. In all cases the total volume of this mixture was 13.1 ml. 2 *N* HCl (0.1 ml) was then added to the mixture, the resultant solution was shaken and placed in the appropriate syringe of the stopped flow instrument. The actual concentration of BNPS in this mixture in any given run was not critical (since excess enzyme conditions were used) and the initial concentration was varied from 0.5×10^{-5} to 1.2×10^{-5} *M* according to the percentage of acetonitrile and the presence or absence of inhibitor. For example, when AcPhe was used as the inhibitor more BNPS was added to the mixture since AcPhe catalyzes the nonenzymatic hydrolysis of the ester. Similarly, increasing the acetonitrile concentration in the syringe retards the spontaneous hydrolysis of BNPS.

Experimental traces were recorded and retained on the screen of a storage oscilloscope, and then photographed. The first few traces of a particular set were discarded and then at least three runs were photographed from each set. pH measurements were

(9) P_1 and P_2 represent either the amino acid contributors to the peptide bond being hydrolyzed or the alcohol moieties of a sulfite ester substrate, and P_3 represents the inorganic sulfite (or bisulfite) liberated during hydrolysis of the latter type of substrate. At the present time it would be only a matter of conjecture to say whether the formation of ES′ from ES leads to the expulsion of P_1, which is the way in which the mechanism is normally invoked, for instance, in chymotrypsin-catalyzed solvolyses. It is also possible that a k_4 step should be added to eq 1. In this step, for example, P_3 might be liberated from an intermediate complex, regenerating the free enzyme.

(10) F. J. Kezdy and M. L. Bender, *Ann. Rev. Biochem.*, **34**, 49 (1965).

(11) M. L. Bender, M. J. Gibian, and D. J. Whelan, *Proc. Natl. Acad. Sci. U. S.*, **56**, 833 (1966).

(12) In the case of AcPheDiBrTyr the K_M constants found at various pH values corresponded fairly well to measurements of K_I using this compound as an inhibitor for the pepsin-catalyzed hydrolysis of diphenyl sulfite (ref 5). However, the uncertainties in the K_M values due to experimental difficulties were considerable, and the K_I/K_M ratios observed were not exactly unity.

(13) T. W. Reid and D. Fahrney, *J. Am. Chem. Soc.*, **89**, 3941 (1967).

(14) M. S. Silver, J. L. Denburg, and J. J. Steffens, *ibid.*, 87, 886 (1965).

(15) K. W. Lo, Ph.D. Thesis, University of Chicago, 1968, p 55.

made on the reaction solutions after each set of runs. The total time elapsed from the addition of BNPS to the substrate syringe until the completion of a set of runs was generally less than 3 min. In all cases if the BNPS was allowed to hydrolyze completely before mixing with the enzyme, no reaction could be observed on the oscilloscope. Also, the transmittance change we observed during any given set of runs was less for later runs than for the initial runs as would be expected since the nonenzymatic hydrolysis of BNPS was proceeding fairly rapidly in the substrate syringe.

The vertical scale of all traces were linear with respect to light transmittance. Reactant concentrations were adjusted to limit the maximum deflection observed to a 5% change in transmittance. Since transmittance changes of this magnitude are approximately proportional to absorbance changes, first-order plots could be constructed directly from the experimental traces.

The hydrolysis of DPS was studied using a Cary Model 15 recording spectrophotometer equipped with a thermostated cell compartment and cuvet holder. The cell compartment was equilibrated to 25.0 ± 0.1°, and the reaction was initiated by substrate addition to a solution containing pepsin and acetonitrile, followed by a mixing period of approximately 30 sec. All runs were followed on the expanded scale slide wire at 269 mμ for approximately 25 min, the enzyme absorbance at this wavelength being blanked out by an appropriate solution in the reference cell. Infinity absorbance values for each run were determined the following day, and the final pH of the solutions for each run was found to be 2.00 ± 0.02.

For the DPS runs pepsin was dissolved in an HCl–KCl solution, pH 2.0, $\mu = 0.1$, containing 1.96% acetonitrile. Acetonitrile was added to each reaction solution to bring the total acetonitrile concentration to a value of 5.13%.

Results

Pepsin-Catalyzed Hydrolysis of BNPS. The nonenzymatic hydrolysis of BNPS in phosphate buffer at pH 2 is too rapid to allow normal substrate in excess conditions to be used for the enzymatic studies. Therefore, we employed concentrations of the enzyme which were in excess over those of the substrate in our kinetic experiments. Under these conditions, the pepsin-catalyzed reaction is far too fast to be followed on a conventional spectrophotometer, and a Durrum–Gibson stopped-flow instrument was employed for the kinetic measurements.

According to the reaction scheme suggested in eq 1, in the presence of a sufficient excess of enzyme, a pseudo-first-order reaction should be observed. The pseudo-first-order rate constants, k_{obsd}, measured are related to the quantities of eq 1 by eq 2 where $k_{cat} = k_2k_3/(k_2 + k_3)$ and $K_M = K_S[k_3/(k_2 + k_3)]$.

$$k_{obsd} = \frac{k_{cat}E}{K_M + E} \qquad (2)$$

A plot of $1/k_{obsd}$ *vs.* $1/E$ should have a slope equal to K_M/k_{cat} and an intercept equal to $1/k_{cat}$. In Figure 1 a plot of the values of $1/k_{obsd}$ *vs.* $1/E$ for the pepsin-catalyzed hydrolysis of BNPS at pH 2 in 0.4% CH_3CN under excess enzyme conditions is given. In all runs a smooth formation of *p*-nitrophenol was observed, indicating that the enzyme-catalyzed hydrolysis does not occur in two kinetically distinguishable steps under the conditions employed. Furthermore, the dependence of the observed rate of hydrolysis on enzyme concentration illustrated in Figure 1 means that we are not simply observing the unimolecular decomposition of the ES′ species. Comparison studies using a Cary Model 15 spectrophotometer showed that the infinity absorbances for the enzyme catalyzed and nonenzymatic hydrolysis of BNPS were the same, indicating that both *p*-nitrophenol molecules are liberated in the enzymatic hydrolysis reaction.

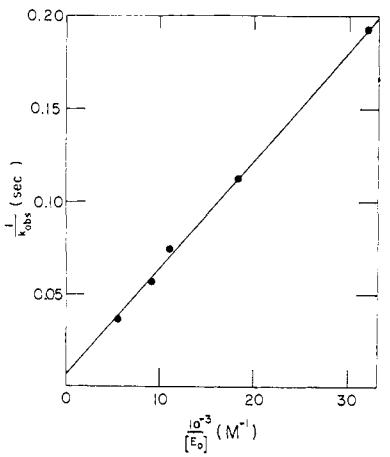


Figure 1. Plot of $1/k_{obsd}$ *vs.* $1/[E_0]$ for the pepsin-catalyzed hydrolysis of BNPS, 25.0 ± 0.1°, pH 2.00 ± 0.02. The solutions for all runs contained 0.4% acetonitrile, and the ionic strength was either 0.02 or 0.08. The plot gives $k_{cat.}$(1/intercept) = 143 sec^{-1} and K_M(slope × $k_{cat.}$) = 8.32 × 10^{-4} *M*.

The kinetic parameters obtained for BNPS from Figure 1 are $k_{cat} = 143$ sec^{-1} and $K_M = 8.32 \times 10^{-4}$ *M*. This k_{cat} value is several orders of magnitude greater than those observed for other pepsin substrates[16] making BNPS the most rapidly hydrolyzed pepsin substrate known to date. The K_M value obtained for BNPS is similar to that observed for DPS hydrolysis at pH 2 (see Conclusions).

Inhibition Studies Using BNPS. The extremely rapid pepsin-catalyzed hydrolysis of BNPS and the comparatively long wavelength absorbance of the liberated *p*-nitrophenol chromophore make this compound ideally suited for obtaining inhibition constants for other less rapidly hydrolyzed sulfite ester substrates. For these determinations if competitive inhibition is assumed, inhibition constants may be obtained from the ratio of observed rate constants in the presence and absence of inhibitor. If $E \ll K_M$, the inhibition constant, K_I, is given by eq 3 where k_{obsd} and k'_{obsd} are the observed

$$K_I = \frac{[I]}{(k_{obsd}/k'_{obsd}) - 1} \qquad (3)$$

pseudo-first-order rate constants in the presence and absence of inhibitor, respectively, but with the same initial enzyme concentrations.[17]

This method was used to determine a K_I value for the pepsin substrate DPS. The hydrolysis of DPS was negligible during the time required to observe the hydrolysis of BNPS. The results of these experiments are presented in Table I. Runs with the same reference number (*e.g.*, 1a and 1b) were performed with the same enzyme solution on the same day. Experiments 3a–3f were performed to determine the inhibition constant for the competitive inhibitor AcPhe as a check on our method. Table II presents the inhibition constants calculated from the data of Table I using eq 3.

The K_I value (7.25 × 10^{-2} *M*) we obtained for inhibition by AcPhe in 10.6% acetonitrile using BNPS as

(16) For a compilation of k_{cat} values for various synthetic substrates of pepsin, see ref 6, pp 46–50 and 98. The values of k_{cat} for various sulfite ester substrates are reported in ref 6, pp 48–49 and 100, and by T. W. Reid, T. P. Stein, and D. Fahrney, *J. Am. Chem. Soc.*, **90**, 7125 (1967).

(17) G. Tomalin, M. Trifunac, and E. T. Kaiser, *ibid.*, **91**, 722 (1969).

Table I. Stopped Flow Inhibition Studies[a]

Expt no.	Inhibitor	% CH_3CN	k_{obsd},[e] sec^{-1}	$10^4 \times I_0$, M
1a[b]		5.3	2.84	
1b	DPS	5.3	2.59	1.67
2a[c]		5.3	2.78	
2b	DPS	5.3	2.55	1.61
2c		10.6	1.38	
3a[d]		0.4	13.58	
3b		5.3	5.26	
3c		10.6	2.16	
3d	AcPhe	0.6	8.23	75.9
3e	AcPhe	5.3	4.01	94.9
3f	AcPhe	10.6	1.91	94.9

[a] All data were gathered at 25°, pH 2.0, μ = 0.027, using BNPS as the substrate and following the hydrolysis of this compound at 325 mμ. [b] Runs 1a and 1b, $E_0 = 3.59 \times 10^{-5}$ M. [c] Runs 2a–2c, $E_0 = 3.5 \times 10^{-5}$ M. [d] Runs 3a–3f, $E_0 = 7.1 \times 10^{-5}$ M. [e] All values of k_{obsd} are average values for three to four determinations.

the substrate is in good agreement with the value of 7.6×10^{-2} M measured by Zeffren and Kaiser in 10% acetonitrile using DPS as the substrate.[5,18] This study with DPS is the only other investigation reported which used a sulfite ester substrate for the determination of the K_I value for AcPhe.

The kinetic constants for the pepsin-catalyzed hydrolysis of DPS at pH 2 in 5.13% acetonitrile were determined under standard substrate in excess conditions. From a plot of E_0/v_0 *vs.* $1/S_0$ values of $k_{cat} = 0.043$ sec^{-1} and $K_M = 5.81 \times 10^{-4}$ M were calculated. A comparison with the K_I value given in Table II in the same solvent system gives a K_I/K_M ratio of 3 for DPS at pH 2. The observation that $K_I \neq K_M$ for DPS is in contrast to the situation found with the peptide inhibitor AcPheDiBrTyr.[5] However, this is the first direct comparison of the experimentally measured inhibition constant with the observed Michaelis constant for an ester substrate of pepsin.

Table II.[a] Inhibition Constants for the Inhibition of BNPS Hydrolysis by DPS and AcPhe at pH 2 and 25°

Inhibitor	% CH_3CN	Calculated $K_I \times 10^4$, M
DPS	5.3	17
DPS	5.3	18
AcPhe	0.4	116
AcPhe	5.3	304
AcPhe	10.6	725

[a] The K_I values obtained for DPS are considerably above the highest concentration of DPS used to inhibit the hydrolysis of BNPS (see Table I). Higher DPS concentrations could not be used because of the limited solubility of this ester. However, although the K_I values for DPS are made somewhat uncertain by the low concentration of DPS employed, our data do clearly indicate that the K_I value must be substantially above the K_M value for DPS.

If K_I is taken to be a true dissociation constant, defined by the relationship shown in eq 4, it can be considered to be equal to the dissociation constant, K_S,[19] in eq 1, and K_M for this scheme would then be given by eq 5.

$$E + I \overset{K_I}{\rightleftharpoons} EI \tag{4}$$

$$K_M = K_I\left(\frac{k_3}{k_2 + k_3}\right) \tag{5}$$

In terms of eq 5 a K_I/K_M ratio of 3 means that $k_3 = 0.5k_2$ at pH 2 and 25° for the pepsin-catalyzed hydrolysis of diphenyl sulfite. In other words the rate constants for the two catalytic steps of eq 1 appear to be of a similar order of magnitude. We believe therefore that the assumption that K_M is a simple dissociation constant which seems to hold for those peptide substrates of pepsin which have been studied carefully cannot be made for all sulfite ester substrates. Of course, the nature of the leaving alcohol moiety is probably very important in determining the relative magnitude of k_2 and k_3 for various sulfite esters. Furthermore, the relative magnitudes of k_2 and k_3 may vary with pH.[20]

From Table I it can be seen that acetonitrile retards the rate of the pepsin-catalyzed hydrolysis of BNPS. Since it is known that the K_M value for DPS hydrolysis at pH 2 in 5% methanol differs from that in 10% acetonitrile while the k_{cat} value is the same in both solvents,[6] it seems reasonable to treat acetonitrile as a competitive inhibitor. By applying eq 3 to runs 3a–3c of Table I and assuming that the solvent in run 3a is completely aqueous (0.4% acetonitrile is actually present), K_I^c values for acetonitrile can be calculated. Such calculations give values of 0.6 and 0.4 M from runs 3b and 3c, respectively. These values should be regarded as approximate since our investigation of inhibition by acetonitrile has not been extensive.

The assumption that acetonitrile inhibition is competitive predicts that for any given competitive inhibitor, the K_I value observed in the presence of acetonitrile should be greater than that in a purely aqueous medium by a factor of $(1 + C/K_I^c)$ where C is the molar concentration of acetonitrile in the former case. Assuming an average K_I^c value of 0.5 M, the trend of increasing K_I values observed for AcPhe in 0, 5, and 10% acetonitrile, respectively, is in satisfactory agreement with that predicted from the application of the above factor. This general trend of larger apparent K_I and K_M values with increased organic solvent concentration has also been observed for AcPheDiBrTyr and DPS.[6]

Conclusions

We have found the BNPS is hydrolyzed by pepsin at pH 2 faster than any other known ester or peptide substrate. The k_{cat}/K_M ratio observed for this reaction is 1.7×10^5 M^{-1} sec^{-1} which is 2300 times larger than the value of 74 M^{-1} sec^{-1} obtained for DPS at the same pH.[21] Despite this large difference in reactivity, both

(18) The spread in literature values of K_I for AcPhe is quite large ($0.2–7.6 \times 10^{-2}$ M) and varies greatly with the temperature, amount of organic solvent present, and particular substrate used. For further discussion of the possible reasons for the variations in the K_I values which have been measured see ref 5 and 6 and W. T. Jackson, M. Schlamowitz, and A. Shaw, *Biochemistry*, 5, 4105 (1966).

(19) The experimental support for assuming the equality of K_S and K_I is strong for the peptide substrates of pepsin which have been examined.[3-5] The assumption that $K_I = K_S$ seems reasonable for sulfite ester substrates but has not really been proven for these compounds.

(20) In ref 11 Reid and Fahrney reported a $k_{cat}^{H_2O}/k_{cat}^{D_2O}$ value of about 1 at pH 4.0 for the pepsin-catalyzed hydrolysis of methyl phenyl sulfite. A possible interpretation of their finding is that the k_2 step of eq 1 is rate determining in the hydrolysis of this sulfite ester at pH 4 and that nucleophilic attack by a group on the enzyme (presumably a carboxylate function) is involved in this step.

(21) The k_{cat}/K_M ratios which are compared have been obtained at different acetonitrile concentrations. The value quoted for BNPS was found in the presence of a very small amount of acetonitrile (0.4%) whereas that given for DPS was obtained in 5.13% acetonitrile. If

esters bind at the same site on the enzyme, as evidenced by the identity of the K_I values measured for the inhibitor AcPhe using either substrate.

A comparison between the k_{cat} and the K_M values observed for the pepsin-catalyzed hydrolysis of BNPS and DPS reveals that the large difference discussed above between the k_{cat}/K_M ratios for the two reactions arises primarily because there is a large difference in the k_{cat} values. In terms of the expression for k_{cat} given in eq 2 we can obtain eq 6 where the primed quantities are those associated with BNPS hydrolysis and unprimed quantities with DPS hydrolysis. It is clear that if $k'_3 \gg k_3$, $k_2 \approx k_3$, and $k'_2 \approx k_2$, the k'_{cat}/k_{cat} ratio would be given by $(k_2 + k_3)/k_3$. Similarly, if $k'_2 \gg k_2$, $k_2 \approx k_3$, and $k'_3 \approx k_3$, $k'_{cat}/k_{cat} = (k_2 + k_3)/k_2$. Since the K_I/K_M ratio obtained in the present study leads to a k_2/k_3 value of 2 the first of these situations would give $k'_{cat}/k_{cat} = 3$, whereas the second would imply that $k'_{cat}/k_{cat} = 1.5$. The fact that the actual value of k'_{cat}/k_{cat} is approximately 4000 means that the presence of the nitro groups in BNPS must cause an acceleration in both the k_2 and k_3 steps of eq 1 relative to the corresponding steps for the hydrolysis of DPS. This is consistent with a mechanism in which 1 mole of *p*-nitrophenol is liberated in the k_2 step of eq 1 when BNPS is hydrolyzed (see ref 9).

$$\frac{k'_{cat}}{k_{cat}} = \frac{(k'_2k'_3)}{(k'_2 + k'_3)}\frac{(k_2 + k_3)}{k_2k_3} \qquad (6)$$

As has been mentioned the apparent K_M values for BNPS and DPS differ much less than the k_{cat} values for the hydrolysis of these compounds. If the K_M values are taken to be a measure of relative binding strength,[22] it is evident that the nitro groups of BNPS have only a slight effect on the binding of this ester to the enzyme.

In contrast to this situation it is known that placing two halogen substituents in the Tyr portion of the peptide substrate AcPheTyr markedly decreases the K_M value for the substituted compound as compared to the unsubstituted compound.[23] It is unclear if this decrease in K_M is due to the electron-withdrawing effects of the halogens or to some other factor.[6,23] If the electron withdrawing effects of substituents are indeed important in the binding of substrates to pepsin, they might be expected to be very pronounced for nitro-substituted pepsin substrates. However, the effect of the nitro groups on the binding of BNPS to pepsin is much smaller than that of the bromo substituents in the case of AcPheDiBrTyr.

K_I^c for acetonitrile is estimated as 0.5 *M*, the "corrected" k_{cat}/K_M ratio for DPS in the absence of acetonitrile would be about 200 M^{-1} sec^{-1} which is smaller than that found for BNPS by a factor of 10^3.

(22) The extent to which the dissociation constant for the Michaelis complex, K_s, is reflected in the apparent K_M values for each substrate is determined by the relative magnitudes of the rate constants k_2 and k_3 (eq 1) for the hydrolysis of that substrate ($K_M = K_Sk_3/[k_2 + k_3]$). However, if $k_3 \geq k_2$, the Michaelis constant still provides a good indication of the strength of the binding of the sulfite ester to pepsin.

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Communications to the Editor

Magnetic Field Effects on Anthracene Triplet–Triplet Annihilation in Fluid Solutions[1]

Sir:

Late in 1968, it became clear from two standpoints that investigating the influence of a magnetic field on the fluid-solution triplet–triplet annihilation process would be very useful. The pioneering work of Johnson, *et al.*,[2] on the effect of a magnetic field on the mutual annihilation of triplet excitons in crystalline anthracene had uncovered some of the more intimate details of that process. Later experimental and theoretical reports also aided an understanding of the interaction.[3-5] As a logical extension, a separate study of the fluid-solution triplet–triplet annihilation reaction promised to provide similar information regarding details of that process. Furthermore, such a study was clearly needed to improve the understanding of the recently reported field effects on electrogenerated chemiluminescence (ECL).[6] We have carried out an investigation of field effects on anthracene triplet–triplet annihilation in fluid solutions, and we report here the major results together with some preliminary conclusions.

The experimental studies are based entirely upon the field dependence of the P-type delayed fluorescence intensity. All measurements were carried out using a phosphorimeter which was constructed especially to operate with the same immersed in a magnetic field.[7] The delayed fluorescence component was isolated by the conventional technique which involves out-of-phase chopping of the excitation and emission beams.[8] The instrument used a C.S. 7-54 glass filter in the excitation beam. Since Pyrex cuvettes were used for all measurements, the sample was excited by light derived mostly from the 365-nm line of the mercury arc employed as an excitation source. The emission beam was filtered by a C.S. 5-57 glass filter. Intensity measurements were recorded using a phase-sensitive detection system. Tests of instrument performance showed it to be completely independent of the magnetic field below 8000 G.

The delayed fluorescence intensity registered from each sample was proportional to the square of the incident excitation intensity, indicating that the emission

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