The pH Dependence of the Pepsin-Catalyzed Hydrolysis of Bis-*p*-nitrophenyl Sulfite

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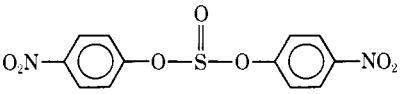
Abstract: The pH dependence of the pepsin-catalyzed hydrolysis of bis-*p*-nitrophenyl sulfite has been determined. The profile found for the pH dependence of k_{cat}/K_M was bell shaped, and the ionization constants calculated for the ionizing groups on the enzyme responsible for this behavior were $pK_{E_1} = 0.82$ and $pK_{E_2} = 5.17$. The value for pK_{E_1} was in good agreement with the corresponding values which have been reported for the majority of peptide substrates hydrolyzed by pepsin. It is more difficult to compare our value for pK_{E_2} to those found for peptide substrates since many of the peptide studies were performed with substrates which are themselves capable of ionization in the pH range 1–5, and in these cases the pK_{E_2} values obtained generally reflect ionization of the substrate. However, our value of pK_{E_2} is in good agreement with that measured from the pH dependence of k_{cat}/K_M for the hydrolysis of at least one neutral peptide substrate Ac-Phe-Phe-NH_2 and in fair agreement with the value reported in the case of Ac-Phe-Tyr-NH_2. The similarity between the k_{cat}/K_M pH profiles we have obtained for bis-*p*-nitrophenyl sulfite and those reported for neutral peptide substrates indicates that there are certain mechanistic features common to both the peptidase and sulfite–esterase activity of pepsin. In terms of the data available in the literature and the usual interpretation of such profiles, a plausible hypothesis is that two carboxyl groups on the free enzyme, one in its acid form and the other in its basic form, are involved in each process.

We have previously reported that bis-*p*-nitrophenyl sulfite (BNPS, I) is an excellent substrate for pepsin, and that the enzyme-catalyzed hydrolysis of this compound at pH 2 to give inorganic sulfite and *p*-nitrophenol proceeds several orders of magnitude more rapidly than that of other known sulfite esters.[3] The fact that a *p*-nitrophenol chromophore is generated during the hydrolysis of BNPS makes it possible to follow this reaction spectrophotometrically at wavelengths where the background absorbance of the enzyme, even when present in great excess, is small.

In order to gain a clearer understanding of the mechanism whereby pepsin catalyzes the hydrolysis of sulfite esters, we decided to undertake a study of the pH dependence of the pepsin-catalyzed hydrolysis of BNPS. Although several such studies using peptide substrates have been reported during the past few years,[4–8] no detailed analysis of the pH dependence of the pepsin-catalyzed hydrolysis of a sulfite ester substrate is available.[9] We therefore felt that such a study with BNPS would provide valuable information about the relationship between the mechanisms of peptide and sulfite hydrolyses. Furthermore, it was attractive to us that BNPS is a neutral substrate in the pH region of pepsin action, and the pK_a's obtained from our study would be, therefore, true reflections of ionizations of the catalytically important groups on the enzyme. The results of our study are reported herein.



I

Experimental Section

Synthesis. The preparation of BNPS has been previously described.[3]

Materials. Pepsin, twice recrystallized and lyophilized, was purchased from Worthington Biochemical Corporation (lot no. PM-8JC.). Enzyme solutions were prepared by dissolving a given amount of pepsin in buffer of the desired ionic strength, and stirring the resultant mixture using a magnetic stirrer for 5–45 min. The duration of this stirring varied with the pH of the buffer used, since the solubility of pepsin generally increases as the solution is made more alkaline. When pepsin concentrations $> 10^{-5}$ *M* were desired, the solutions were then filtered through a Millipore filter with a pore size of 0.22 μ, which had been sterilized immediately before use. The pH values of the solutions were then adjusted as desired, and the enzyme concentrations were determined spectrophotometrically at 278 mμ on a Cary-15 recording spectrophotometer, using ϵ_{278} 51,500.[10] Enzyme solutions were stored at 4°, and were generally used within 24 hr of their preparation. Enzyme stock solutions at pH 3.5 or above were always used within several hours of their preparation, and never stored.

Acetonitrile, deionized water, and stock BNPS solutions were obtained or prepared as previously described.[3] pH measurements were taken on a Radiometer pH meter, Model 4C, using various combined, concentric calomel-glass electrodes. The meter was standardized with an appropriate standard buffer (Fisher Certified) before each determination.

A solution of a given buffer at a given pH was usually prepared by mixing two solutions in the necessary proportions, one containing only the protonated buffer species, and the other containing only the unprotonated buffer species. The desired ionic strength was attained either by diluting this mixture to an appropriate final volume, or by adding NaCl. The compositions of the buffer used at various pH values were as follows: pH 1.15–1.91, HCl–NaCl solution; pH 2.77–3.47, phosphate; pH 3.47 and 4.00, acetate; pH 4.95 and 5.36, 2-(*N*-morpholino)ethane sulfonic acid (MES). All of these buffers were prepared at $\mu = 0.05$ except for

(1) Predoctoral Fellow of the National Science Foundation, 1967–1970. This report is taken, in part, from the Ph.D. Thesis of S. W. May, University of Chicago, Dec 1970.

(2) Fellow of the Alfred P. Sloan Foundation, 1968–1970.

(3) S. W. May and E. T. Kaiser, *J. Amer. Chem. Soc.*, **91**, 6491 (1969).

(4) E. Zeffren and E. T. Kaiser, *ibid.*, **89**, 4204 (1967).

(5) G. E. Clement, S. L. Snyder, H. Price, and R. Cartmell, *ibid.*, **90**, 5603 (1968).

(6) G. E. Clement, J. Rooney, D. Zakheim, and J. Eastman, *ibid.*, **92**, 186 (1970).

(7) A. J. Cornish-Bowden and J. R. Knowles, *Biochem. J.*, **113**, 353 (1969).

(8) J. L. Denburg, R. Nelson, and M. S. Silver, *J. Amer. Chem. Soc.*, **90**, 479 (1968).

(9) A sigmoidal k_{cat} *vs.* pH profile for the pepsin-catalyzed hydrolysis of methyl phenyl sulfite has been reported (see Discussion): T. W. Reid and D. Fahrney, *ibid.*, **89**, 3941 (1967).

(10) (a) M. S. Silver, J. L. Denburg, and J. J. Steffens, *ibid.*, **87**, 886 (1965); (b) G. E. Perlmann, *Proc. Nat. Acad. Sci. U. S.*, **45**, 915 (1959).

Table I. Kinetic Parameters for the Pepsin-Catalyzed BNPS Hydrolysis at Various pH Values[a,b]

pH	k_{cat}, sec^{-1}	$10^4 \times K_M$, M	$10^{-5} \times k_{cat}/K_M$, M^{-1} sec^{-1}	Buffer[c]	Ionic strength of reaction solution[d,e]
0.70	41.5 ± 9.4	3.66 ± 0.86	1.13 ± 0.07	H(1)	0.2
1.15[f]			1.80 ± 0.01	H(2)	0.08
1.50	60 ± 5	2.53 ± 0.23	2.37 ± 0.09	H(3)	0.045
1.91[g]	56.9 ± 7	2.19 ± 0.31	2.60 ± 0.17	H(3)	0.033
2.77	70 ± 4	2.75 ± 0.18	2.54 ± 0.07	P	0.028
3.00	70 ± 5	2.56 ± 0.22	2.75 ± 0.11	P	0.026
3.47	72.6 ± 4.1	2.83 ± 0.18	2.56 ± 0.08	P, A	0.025
4.00	53 ± 2	2.05 ± 0.12	2.59 ± 0.08	A	0.025
4.95	24 ± 0.5	1.54 ± 0.04	1.56 ± 0.03	M	0.025
5.36	17.5 ± 1.4	1.45 ± 0.16	1.20 ± 0.09	M	0.025

[a] All experiments were performed at 25.0 ± 0.1° in 0.4% acetonitrile. [b] Values of the kinetic parameters were calculated from the experimental data by use of a high-speed computer program described in the Experimental Section. Error limits are values for one standard deviation. The error in k_{cat} reflects the error in the intercept, while that in K_M is weighted summation of the errors in the slope and intercept. [c] H(1) is 0.2 M HCl, measured at pH 0.75; H(2) is HCl–NaCl, μ = 0.08; H(3) is HCl–NaCl, μ = 0.05; P is phosphate, μ = 0.05; A is acetate–NaCl, μ = 0.05; M is MES, μ = 0.05. After mixing with the substrate solutions, the ionic strengths shown in Table I were attained for the reaction solutions. [d] At pH 3.00 and below, the hydrogen ion concentration has been taken into account in calculating the ionic strength. [e] This takes into account the HCl added to the substrate syringe (see Experimental Section). [f] Due to a shortage of the same lot of pepsin only enough points for a meaningful determination of k_{cat}/K_M were gathered at this pH. [g] The values of the kinetic parameters reported here differ somewhat from those which we have reported earlier at pH 2.[3] However, the double reciprocal plot from which the earlier values were obtained was hand drawn, while the present values were obtained from the weighted least-squares computer treatment of the raw data. In addition, a different lot of commercial pepsin and different buffer system were used in the previous study.

the HCl–NaCl solution at pH 1.15, which had μ = 0.08. In addition, the data at pH 0.70 were obtained using a 0.2 M hydrochloric acid solution, which actually gave a pH meter reading of 0.77 when the meter was standardized against pH 1 standard buffer (*cf.* the similar observations reported in ref 8).

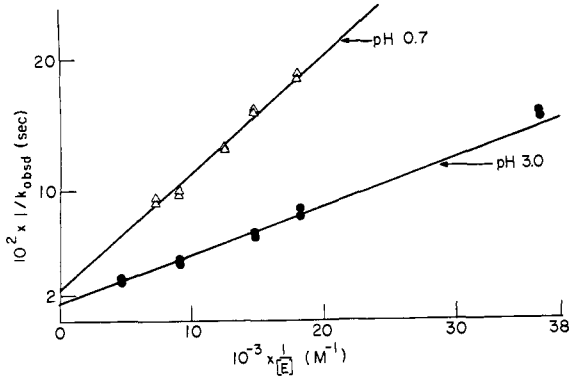


Figure 1. Plots of $1/k_{obsd}$ *vs.* 1/[E] for the pepsin-catalyzed hydrolysis of BNPS at 25 ± 0.1° and in 0.4% acetonitrile: pH 0.70, △; pH 3.00, ●. The lines are computer calculated, weighted least-squares fit of the data to eq 2 at each pH.

Kinetic Measurements. The pepsin-catalyzed hydrolysis of BNPS was studied on a Durrum-Gibson stopped-flow spectrophotometer using the same general procedure which we have described previously,[3] except that the amount of HCl added to the substrate syringe was varied according to the pH of the experiment. This caused slight variations in the ionic strength of the final reaction solutions, but control runs at both pH 1.5 and 2.0, as well as the results of Jackson, *et al.*,[11] and of Zeffren and Kaiser[4] indicate that in this range, the effects of small variations in ionic strength are negligible. (The final ionic strengths and buffer compositions at various pH values are tabulated in the Results.) The purpose of running experiments at pH 3.47 in both phosphate and acetate buffers was to determine whether a smooth transition between these buffers could be obtained.

Kinetic Schemes. The probable reaction sequence for the pepsin-catalyzed hydrolysis of sulfite esters is represented by eq 1.[3,12-15]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_1 + P_2 + P_3 \qquad (1)$$

According to this scheme, in the presence of a sufficient excess of enzyme, a pseudo-first-order reaction should be observed, with its experimentally measured pseudo-first-order rate constant, k_{obsd}, related to the parameters of scheme 1 by eq 2

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

where

$$k_{cat} = \frac{k_2k_3}{k_2 + k_3} \qquad (3)$$

and, for $k_{-1} \gg k_2$

$$K_M = K_s[k_3/(k_2 + k_3)] \qquad (4)$$

(K_s is defined as k_{-1}/k_1). According to eq 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 $1/k_{cat}$. Thus, such a double reciprocal plot allows evaluation of each of the kinetic parameters separately. (See Figure 1 and Table I.)

The time course of the pepsin-catalyzed hydrolysis of BNPS follows first-order kinetics (approximately four half-lives) at all pH values, in the solvents listed above. However, in some other solvent systems, complicating solvent effects were encountered, and these will be discussed in a subsequent communication from this laboratory.[14]

For analyzing the pH dependence of the pepsin-catalyzed hydrolysis of BNPS, two different schemes were employed. The first of these is that assumed in the computer program for pH–rate profiles (see below) used to determine the k_{cat}/K_M *vs.* pH curve of Figure 2, and one of the k_{cat} *vs.* pH curves of Figure 3, and

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

(12) The abbreviations used in this paper are defined in ref 3.

(13) P_1 and P_2 represent the alcohol moieties of the sulfite ester substrate and P_3 represents inorganic bisulfite (or SO_2) liberated during the hydrolysis of the ester. At the present time it is only a matter of conjecture to say whether the formation of ES′ from ES leads to expulsion of P_1, which is the way in which the mechanism is normally invoked, for instance, in chymotrypsin-catalyzed solvolyses. The question concerning when the liberation of inorganic sulfite occurs is considered elsewhere (ref 14 and 15).

(14) S. W. May and E. T. Kaiser, in preparation. In MES buffer, as well as in some other solvent systems, a relatively slow, solvent-associated reaction is observed, following the completion of the more rapid enzymatic reaction. We have studied this phenomenon in considerable detail, and our results are discussed amply elsewhere (S. W. May and E. T. Kaiser, submitted for publication). However, it seems appropriate to mention here that while the rate of this "slow" reaction is dependent upon the solvent used, it is independent of enzyme concentration. Moreover, substrate-in-excess experiments with BNPS have revealed that the "slow" reaction does not represent decomposition of a covalent ES′ species at the active site of pepsin.

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

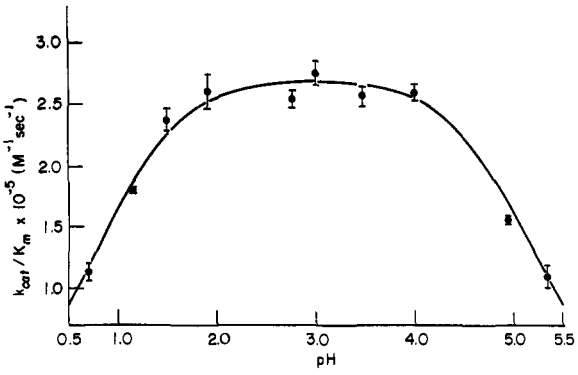


Figure 2. Plot of k_{cat}/K_M *vs.* pH for the pepsin-catalyzed hydrolysis of BNPS at 25°, aqueous solution (0.4% acetonitrile). The curve is a theoretical one for $pK_1 = 0.82$, $pK_2 = 5.17$, $(k_{cat}/K_M)_{lim} = 2.71 \times 10^5$ M^{-1} sec^{-1}, and is a computer calculated least-squares fit to the data. Experimental points and one standard deviation are shown.

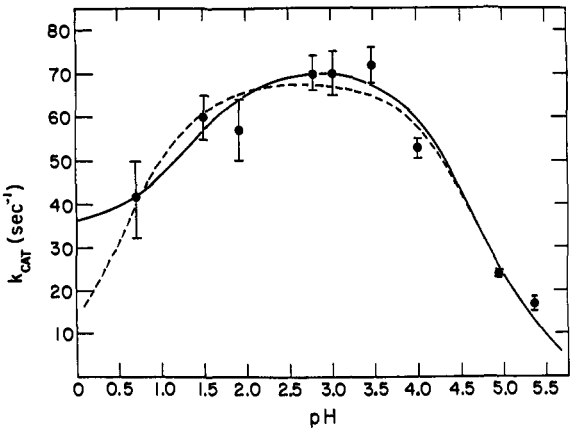
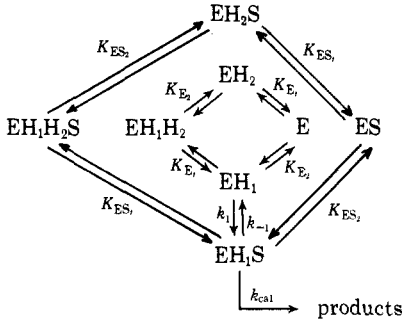


Figure 3. Plot of k_{cat} *vs.* pH for the pepsin-catalyzed hydrolysis of BNPS at 25° in aqueous solution (0.4% acetonitrile). Experimental points and one standard deviation are shown: ----, theoretical curve assuming k_{cat} goes to zero in acid solution; ——, theoretical curve assuming k_{cat} levels in acid solution. Parameters for the curves are given in Table II.

is illustrated below. As shown, this scheme assumes that there are two catalytically important ionizing groups in pepsin, that only the EH_1 form of the enzyme binds to the substrate, and that only the EH_1S form of the enzyme–substrate complex goes on



(5)

to products. Also, it is implicity assumed that the ionizations shown occur much faster than either complex formation or catalysis. The scheme recognizes that the two ionizations are independent of each other and assumes that the state of ionization of one group has no effect on the pK_a of the other group. Finally, it is assumed that the state of ionization of the *substrate* is the same throughout the pH range of interest—an assumption which is obviously valid for sulfite esters.

Equation 5 gives rise to the following equations for the predicted pH dependence of the kinetic parameters.

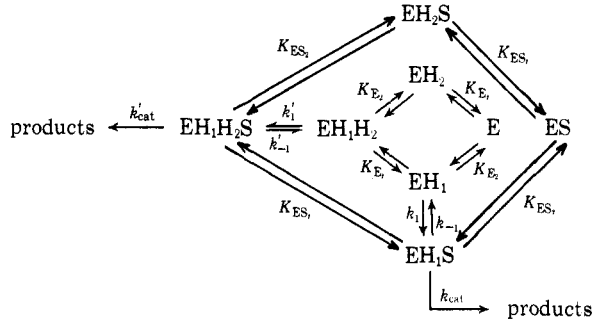
$$k_{cat} = \frac{(k_{cat})_{lim}}{1 + \frac{H}{K_{ES_1}} + \frac{K_{ES_2}}{H} + \frac{K_{ES_2}}{K_{ES_1}}} \quad (6)$$

$$k_{cat}/K_M = \frac{(k_{cat}/K_M)_{lim}}{1 + \frac{H}{K_{E_1}} + \frac{K_{E_2}}{H} + \frac{K_{E_2}}{K_{E_1}}} \quad (7)$$

$$K_M = (K_M)_{lim}\left[\frac{1 + \frac{H}{K_{E_1}} + \frac{K_{E_2}}{H} + \frac{K_{E_2}}{K_{E_1}}}{1 + \frac{H}{K_{ES_1}} + \frac{K_{ES_2}}{H} + \frac{K_{ES_2}}{K_{ES_1}}}\right] \quad (8)$$

The second scheme which was employed is similar to scheme 5 except that it postulates two active forms of the enzyme, $EH_1(EH_1S)$ and $EH_1H_2(EH_1H_2S)$. This is illustrated in eq 9.

Scheme 9 gives rise to the expression for the theoretical pH dependence of the catalysis constant, k_{cat}, which is given in eq 10.



(9)

$$k_{cat} = \frac{[(k_{cat}')_{lim}]\left[\frac{H}{K_{ES_1}}\right] + (k_{cat})_{lim}}{1 + \frac{H}{K_{ES_1}} + \frac{K_{ES_2}}{H} + \frac{K_{ES_2}}{K_{ES_1}}} \quad (10)$$

Equation 10 was used to calculate the appropriately labeled curve in Figure 3. As shown, this equation predicts a leveling of k_{cat} in acid solution, as opposed to the simple bell-shaped dependence predicted by eq 6.

The expression describing the pH dependence of k_{cat}/K_M predicted from scheme 9 has the same general form as eq 10, and thus also predicts a leveling of this parameter in acid solution. However, our data indicate that the pH dependence of k_{cat}/K_M is best described by a simple bell-shaped curve (see below). A discussion of how this result can be accommodated by scheme 9 is presented in the Appendix.

Treatment of Data. The kinetic constants for BNPS hydrolysis at various pH values were calculated from the experimental rate data using a Fortran IV computer program with an IBM Model 360/65 computer. This program computes a weighted, least-squares fit of the data to the reciprocal form of eq 2. The general equations and weighting factors used in writing this program were similar to those described by Wilkinson.[16]

The high-speed computer program used to fit the kinetic data to eq 6 and 7 has been described by Hall.[17]

Results

The fact that construction of double reciprocal plots based on eq 2 allows separation and evaluation of individual kinetic parameters enabled us to undertake a detailed study of the pH dependence of the pepsin-catalyzed hydrolysis of BNPS. In these experiments,

(16) G. N. Wilkinson, *Biochem. J.*, 80, 324 (1960).
(17) P. L. Hall, Ph.D. Thesis, University of Chicago, Chicago, Ill., 1967.

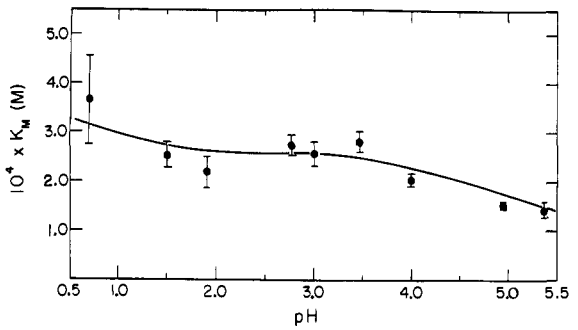


Figure 4. Plot of K_M *vs.* pH for the pepsin-catalyzed hydrolysis of BNPS at 25°, in aqueous solution (0.4% acetonitrile). Experimental points and one standard deviation are shown.

the rates of BNPS hydrolysis at various enzyme concentrations and a given pH were measured using the stopped-flow instrument, and a plot of $1/k_{obsd}$ *vs.* 1/[E] was constructed. The kinetic parameters, k_{cat}, K_M, and k_{cat}/K_M were evaluated from the slope and intercept of this plot. This entire procedure was repeated at various intervals of pH, and the results were then combined to give the pH dependence of each of the individual kinetic parameters. Some typical double reciprocal plots are illustrated in Figure 1, and Table I provides a complete tabulation of the kinetic parameters obtained from such plots at various intervals of pH.

The data on BNPS hydrolysis were gathered in aqueous media containing only 0.4% CH_3CN. It was possible to use such small concentrations of organic solvent since excess enzyme conditions were employed, and the actual substrate concentrations were always very small (less than 10^{-5} *M*). Normally, however, enzymatic reactions are studied under excess substrate conditions, and the insolubility of aromatic sulfite esters in aqueous media severely limits the maximum attainable substrate concentration in such studies. This, in turn, necessitates a very large extrapolation to determine the intercept in double reciprocal plots, and prevents accurate evaluation of the individual kinetic parameters. For this reason, Reid and Fahrney[9] report only the value of k_{cat}/K_M for DPS hydrolysis in aqueous solution. Zeffren[18] employed 5–10% organic solvent to increase the solubility of DPS, and we have been forced to do the same in some experiments which are described elsewhere.[3,14] Unfortunately, such organic solvents are known to inhibit pepsin-catalyzed hydrolyses[3,7,19] and thereby often complicate the interpretation of kinetic data. It is thus evident that excess enzyme conditions are desirable in studying the pepsin-catalyzed hydrolyses of aromatic sulfite esters.

It is noteworthy that above pH 2.77 it was possible to attain final enzyme concentrations approaching K_M for BNPS. Moreover, at pH 4.00, 4.95, and 5.36, enzyme concentrations well above K_M were attained. On the other hand, the decreased solubility of pepsin below pH 2.5 generally limited the maximum attainable enzyme concentrations to about 65–70% of the observed K_M. These considerations introduced relatively greater uncertainty into the values of the intercepts of plots of $1/k_{obsd}$ *vs.* 1/[E] below pH 2.5, and this is reflected in the larger standard errors in k_{cat} and K_M.

(18) E. Zeffren, Ph.D. Thesis, University of Chicago, Chicago, Ill., 1967.

(19) J. Tang, *J. Biol. Chem.*, 240, 3810 (1967).

The values of k_{cat}/K_M, obtained from the slopes of double reciprocal plots, are unaffected by such solubility considerations.

There is, at present, no active-site titrant available for pepsin, and enzyme concentrations must be estimated spectrophotometrically. This means that the value of the intercept of a plot of $1/k_{obsd}$ *vs.* 1/[E] will vary according to the percentage of active enzyme actually present in the pepsin preparation used. For this reason, the same lot of commercial pepsin (Worthington, lot no. PM 8JC) was used throughout in gathering data on the pH dependence of BNPS hydrolysis. Some control experiments with another lot of commercial pepsin (Worthington, lot no. PM-OAB) at pH 1.50 indicated that its specific activity toward BNPS was similar to that of the first lot. However, since commercial pepsin is probably heterogeneous, it is likely that studies with activated pepsin, or with other commercial lots, will yield somewhat different values for k_{cat} and K_M.[20]

Figures 2, 3, and 4 present plots of the pH dependence of the kinetic parameters k_{cat}/K_M, k_{cat}, and K_M, respectively. The theoretical curve in Figure 2 is a computer-calculated fit of the data to eq 7. In Figure 3, two theoretical curves are shown: one calculated using eq 6 and the other using eq 10. The pK values and limiting parameters associated with these figures are tabulated in Table II. It is apparent that the k_{cat}/K_M profile is a well-defined bell-shaped curve, while the K_M profile is essentially flat in the region of our study. The relatively large error limits in many of the K_M values may cause a masking of some subtleties in this latter profile, and, in particular, they may preclude a clear determination whether the apparent rise of K_M at low pH is significant. As shown in Figure 3, we have presented two possible theoretical curves for our k_{cat} *vs.* pH profile, one of which gives a simple bell-shaped curve, while the other levels in acid solution. The relatively large standard errors of the k_{cat} values in the acidic region preclude a clear choice between these two possibilities. This situation was unavoidable, however, due to the decreased solubility of pepsin below pH 2.5, which necessitated relatively large extrapolations in our double reciprocal plots, and in turn, caused generation of larger

Table II. Limiting Parameter Values and pK Values Obtained from Studies on the pH Dependence of the Pepsin-Catalyzed Hydrolysis of BNPS[a]

Profile	Limiting value	pK_1	pK_2
k_{cat}/K_M	2.71×10^5 M^{-1} sec^{-1}	0.82	5.17
k_{cat} (to zero)[b]	68.6 sec^{-1}	0.57	4.73
k_{cat} (acid leveling)[c,d]	(Acid form) 35 sec^{-1} (Ionized form) 72 sec^{-1}	1.35	4.73
K_M[e]			

[a] All data gathered at 25.0 ± 0.1° and in 0.4% acetonitrile. [b] Assuming parameter tends to zero in acid solution. [c] Assuming parameter tends to level at an appreciable value in acid solution. [d] Acid portion of curve was hand calculated (see Figure 3). [e] Within the accuracy of our data, this parameter appears to be independent of pH in the range 0.7–4.0 and perhaps from pH 0.7 to 5.4.

(20) Several investigators have reported varying differences in activities between commercial and activated pepsin. For example, see (a) T. G. Rajagopalan, S. Moore, and W. H. Stein, *ibid.*, 241, 4940 (1966); (b) R. S. Bayliss, J. R. Knowles, and G. B. Wybrandt, *Biochem. J.*, 113, 377 (1969); (c) ref 6.

standard errors from the computer treatment. However, careful inspection of Figure 3 reveals that the curve which levels in acid solution also gives a better fit to the data in the pH 2.75–3.5 region. This is a consequence of the fact that in the calculation of the latter curve a pK_1 value was assumed which is about 0.75 unit higher than that used in calculating the simple bell-shaped curve.

It has been pointed out above that scheme 9, which forms the theoretical basis for the leveling k_{cat} curve, assumes that both EH_1H_2S and EH_1S are active toward BNPS. An alternative explanation for this leveling behavior of k_{cat} arises from the fact that if an ES′ species is indeed formed in BNPS hydrolysis, then k_{cat} is actually a complex constant defined by the relationship $k_{cat} = k_2k_3/(k_2 + k_3)$ (see eq 3). Thus, the k_{cat} *vs.* pH profile may be partially reflecting the pH dependence of the k_3 step, as well as that of the k_2 step, a fact which is ignored by both schemes 5 and 9. As one goes from low to high pH there may be a shift in the relative magnitudes of k_2 and k_3, so that, at various intermediate pH values, k_{cat} may equal k_2, k_3, or a combination of the two. Obviously then the situation regarding the pH dependence of k_{cat}, at least on the acidic side of Figure 3, may be extremely complex.

Actually, it is this complexity in k_{cat} which often makes k_{cat}/K_M the most meaningful parameter, in cases where the k_2 and k_3 steps cannot be separated kinetically. For such cases, since $k_{cat} = k_2k_3/(k_2 + k_3)$ and $K_M = K_s[k_3/(k_2 + k_3]$, it is apparent that k_{cat}/K_M is equivalent to k_2/K_s. This means that, in terms of the usual interpretation of pH profiles, the pH dependence of this parameter always reflects simply the important ionizations of the free enzyme.

Discussion

Sulfite esters are nonspecific pepsin substrates with structures which are obviously quite different from those of the more "normal" peptide substrates of this enzyme. Yet, these compounds have certain features which make them attractive for use in mechanistic studies. They are uncharged (neutral) in the pH region of pepsin activity, and they are highly sensitive substrates, in the sense that they are rapidly hydrolyzed by pepsin. Moreover, the enzymatic hydrolysis of these compounds can usually be followed spectrophotometrically without undue complications. It is therefore important to investigate the details of the catalytic process operative in the enzymatic hydrolysis of sulfite esters, and to define the relationship between the peptidase and sulfite–esterase activities of pepsin. In this regard, the information obtained from pH–rate studies with various pepsin substrates can be of considerable value.

The data in Tables I and II represent the first detailed pH–rate study available for a sulfite ester substrate of pepsin. Reid and Fahrney[9] have reported a sigmoidal k_{cat} *vs.* pH profile for methyl phenyl sulfite, but their study was confined to the very limited range of pH 2–4. In addition, they did not report the pH dependencies of K_M and k_{cat}/K_M. The pK_{E_1} value of 0.82 obtained from our k_{cat}/K_M profile is in good agreement with the value of about 1.0 obtained from the majority of the reported studies on peptide substrates of pepsin.[4,7,8] There are considerably less data available with which to compare our pK_{E_2} value, since many of the peptide studies were performed with substrates which are themselves capable of ionization in the pH range 1–5, and in these studies the pK_{E_2} values obtained from the appropriate k_{cat}/K_M profiles generally reflect ionization of the substrate. However, our value of 5.17 is in quite good agreement with that of 4.75 obtained for Ac-Phe-Phe-NH_2 by Cornish-Bowden and Knowles,[7] while both of these values are somewhat higher than that of 4.35 obtained by Denburg, *et al.*,[8] for Ac-Phe-Tyr-NH_2.

The similarity between the k_{cat}/K_M profile we have obtained for BNPS and those reported for neutral peptide substrates indicates that there are certain mechanistic features common to both the peptidase and sulfite–esterase activity of pepsin. In terms of the usual interpretation of such profiles, two carboxyl groups on the free enzyme, one active as an acid and the other as a base, are involved in each process. This similarity becomes all the more striking when one considers the fact that BNPS is hydrolyzed by pepsin several orders of magnitude more rapidly than are the neutral peptide substrates. Interestingly, Fruton and coworkers[21,22] have recently reported that highly sensitive, cationic peptide substrates exhibit a pH behavior which is quite different from that observed with relatively resistant peptide substrates. In view of our results with BNPS, it appears that factors other than relative reactivity, *e.g.*, the presence or absence of a charged group on the substrate, may significantly influence the pH behavior of a given pepsin substrate.

If K_M is taken as a rough indication of substrate binding, the fact that K_M for BNPS is relatively invariant with pH implies that pepsin does not undergo any *gross* conformational change which affects the integrity of the active site in region pH 1.5–5.[23] Knowles, *et al.*,[24] have drawn a similar conclusion from the fact that K_I values for uncharged dipeptide inhibitors of pepsin are also independent of pH in this region.

Turning to the k_{cat} profile, it is noteworthy that the pK_{ES_1} value of 0.57 obtained from the simple bell-shaped curve of Figure 3 is much lower than most other reported values, while that obtained from the leveling curve of this figure (1.35) is in excellent agreement with the values obtained in other investigations. The pK_{ES_2} value obtained from either of these two curves is the same (4.73), and is similar to other reported values. In addition, the similarity of pK_{E_2} and pK_{ES_2} implies that these are dissociation constants for the same group with the slight shift being caused by binding of the substrate. However, it must be remembered that, as mentioned above, there are certain complexities in the interpretation of this profile. In particular, more information is needed regarding the rate-determining step and the nature of the intermediates formed during the pepsin-catalyzed hydrolysis of sulfite esters, before a clear understanding of the meaning of k_{cat} can be obtained. To this end, a series of studies have been carried out in this laboratory, the results of which will be reported elsewhere.[14]

(21) G. P. Sachdev and J. S. Fruton, *Biochemistry*, **8**, 4231 (1969).
(22) G. P. Sachdev and J. S. Fruton, *ibid.*, **9**, 4465 (1970).
(23) The extent to which the dissociation constant for the Michaelis complex, K_s, is reflected in the apparent K_M value, is determined by the relative magnitude of k_2 and k_3 (eq 1). However, if $k_3 > k_2$, K_M still provides a good indication of the strength of substrate binding.
(24) J. R. Knowles, H. Sharp, and P. Greenwell, *Biochem. J.*, **113**, 343 (1969).

On the basis of chemical modification[20b,25-28] and dephosphorylation[6,29] studies, it is generally assumed that the bell-shaped k_{cat} and k_{cat}/K_M profiles obtained from pH–rate studies on pepsin substrates reflect the ionizations of two catalytically important carboxyl groups on the enzyme. However, it has recently been suggested[30,31] that there may be several closely spaced carboxyl groups, with overlapping pK values ranging from about 1 to 5, near the active site of pepsin. Since our values of pK_{E_2} and pK_{ES_2} are somewhat higher than those obtained from studies on peptide substrates, the possibility must be considered that our values reflect the ionization of a different carboxyl group than that involved in the hydrolysis of peptide substrates. Moreover, since the chemical modification studies of Lundblad and Stein[30] implicate a carboxyl group on the free enzyme with a pK near 5, it seems possible that this is the same group which is involved in BNPS hydrolysis. However, such conclusions must be regarded as tentative until more data on sulfite ester hydrolyses become available.

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Appendix

According to scheme 9 which postulates that both the EH_1S and EH_1H_2S forms of the Michaelis complex go to products, the expression which describes the pH dependence of k_{cat}/K_M for the pepsin-catalyzed hydrolysis of BNPS is given by eq I. This expression has the

$$\frac{k_{cat}}{K_M} = \frac{\left[\frac{k_{cat}'}{K_s'}\right]_{lim}\left(\frac{H}{K_{E_1}}\right) + \left[\frac{k_{cat}}{K_s}\right]_{lim}}{1 + \frac{H}{K_{E_1}} + \frac{K_{E_2}}{H} + \frac{K_{E_2}}{K_{E_1}}} \qquad (I)$$

same general form as eq 10 and thus predicts a leveling of k_{cat}/K_M in acid solution. However, our data seem to indicate that the pH dependence of k_{cat}/K_M is best described by a simple bell-shaped curve (see Figure 2). This result can be accommodated by eq I if one assumes that in the range of our study, $[k_{cat}/K_s]_{lim} \gg [k_{cat}'/K_s']_{lim}(H/K_{E_1})$. Thus, to a good approximation, the theoretical pH dependence of k_{cat}/K_M will be described by eq 7, *i.e.*, it will be the same as that predicted from scheme 5.

In theory, the relative magnitudes of $[k_{cat}/K_s]_{lim}$ and $[k_{cat}'/K_s']_{lim}(H/K_{E_1})$ can be estimated using the k_{cat}/K_M *vs.* pH and the k_{cat} *vs.* pH profiles obtained in our study (Figures 2 and 3, respectively). These profiles afford values for $[k_{cat}']_{lim}$, $[k_{cat}]_{lim}$, K_{E_1}, and K_{ES_1}, and since for scheme 9, $K_{ES_1}K_s = K_{E_1}K_s'$, the relative magnitudes of $[K_s]_{lim}$ and $[K_s']_{lim}$ can then be obtained. Unfortunately, the relatively large standard errors in our k_{cat} data on the acidic side of the pH profile shown in Figure 3 introduce considerable uncertainty into the values of $[k_{cat}']_{lim}$ and K_{ES_1} obtained from these data, and we therefore cannot estimate the relative magnitudes of $[k_{cat}'/K_s']_{lim}$ and $[k_{cat}/K_s]_{lim}$ to any degree of accuracy.

Communications to the Editor

A Simple Approach to the Tetracyclo[3.3.0.0^{2,4}.0^{3,6}]oct-7-ene System. 7,8-Diazatetracyclo[3.3.0.0^{2,4}.0^{3,6}]oct-7-ene

Sir:

Studies of the $(CH)_8$ energy surface have led to great interest into the independent synthesis of the various $(CH)_8$ isomers to allow entry onto the surface at various points.[1] Recently, a rather tedious synthesis of a carboxylic acid possessing the tetracyclo[3.3.0.0^{2,4}.0^{3,6}]oct-7-ene ring system was reported.[2] We wish to report a simple approach to molecules of this structural variety. Specifically, we report the synthesis of the related nitrogen-substituted compound **1**, a molecule of considerable interest as a potential precursor of the unknown parent prismane molecule.

Scheme I summarizes the synthetic sequence. Alkylation of sodium cyclopentadienide[3] with *N*-bromomethylbenzamide[4] at −50° followed by Diels–Alder cycloaddition with dimethyl azodicarboxylate at 0° produced dimethyl 7-*syn*-benzamidomethyl-2,3-diazabicyclo[2.2.1]hept-5-ene-2,3-dicarboxylate (**2**)[5] in 78% yield. Stereochemistry at the 7 position was initially assumed to be syn based on least hindered approach

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(5) All new compounds had satisfactory analytical and spectral data.