The Reactions of Sultones with Chymotrypsin. The pH Dependence of Sulfonylation and Desulfonylation

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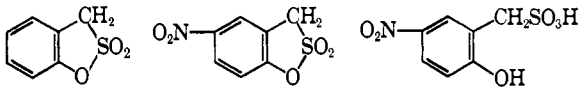
Abstract: Five-membered cyclic sulfonates have been found to be highly reactive toward chymotrypsin. An examination by the stopped-flow technique of the kinetics of sulfonylation of α-chymotrypsin by 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone shows that the function k_2/K_s has a bell-shaped pH dependence. Ionizing groups with pK's of 7.0 and 8.7 appear to be implicated in the sulfonylation reaction. The interesting observation has been made that the sulfonyl enzyme formed, 2-hydroxy-5-nitro-α-toluenesulfonyl-α-chymotrypsin, desulfonylates over a considerable pH range at rates which are conveniently measurable with a conventional spectrophotometer. Analysis of the pH dependence of this desulfonylation reaction strongly suggests that the 2-hydroxyl group is catalytically important in the decomposition of the sulfonyl enzyme.

In recent investigations we have found that certain five-membered cyclic sulfur-containing esters are exceptionally labile to alkaline attack.[2-4] For example, the five-membered cyclic sulfonate I, 2-hydroxy-α-toluenesulfonic acid sultone, undergoes hydroxide ion catalyzed hydrolysis 10^6 times faster than its open-chain analog, phenyl α-toluenesulfonate.[3] An examination of the reactivity of this and related cyclic esters toward several enzymes suggests that these compounds may prove to be very useful reagents for investigations on enzymatic reaction mechanisms.[5,6]



I II III

A preliminary study has shown that the nitrosultone II reacts with the well-characterized proteolytic enzyme α-chymotrypsin (CT) in a rapid, stoichiometric reaction to form a catalytically inactive sulfonyl enzyme. This sulfonyl enzyme subsequently decomposes in a slow first-order reaction to give the active enzyme and the product acid III.[5] These observations suggested that compound II might be reacting with CT much like a normal ester or amide substrate with a reaction sequence like the following

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EP \xrightarrow{k_3} E + P \qquad (I)$$

where E represents the enzyme, S is the sultone, ES is a noncovalent complex of the two species, EP is a covalent intermediate, and P is the product acid III.

At this time we report the results of a kinetic study of the sulfonylation and desulfonylation reaction. We will also propose a mechanistic interpretation of these reactions and present certain further observations which support this interpretation.

(1) Predoctoral Fellow of the National Science Foundation.
(2) E. T. Kaiser, I. R. Katz, and T. F. Wulfers, *J. Am. Chem. Soc.*, 87, 3781 (1965).
(3) O. R. Zaborsky and E. T. Kaiser, *ibid.*, 88, 3084 (1966).
(4) K. Kudo, O. R. Zaborsky, and E. T. Kaiser, *ibid.*, 89, 1393 (1967).
(5) J. H. Heidema and E. T. Kaiser, *ibid.*, 89, 460 (1967).
(6) K. W. Lo and E. T. Kaiser, *Chem. Commun.*, 834 (1966).

Kinetics of the Sulfonylation Reaction

When a solution of sultone II is added to a solution of CT at pH values near neutrality, a "burst" in absorbance is observed with a λ_{max} of 3910 Å. Subsequent to the burst a slow rise in absorbance is observed with a coincident shift of the λ_{max} to 4110 Å. These observations have been shown to be the result of a very rapid sulfonylation of the enzyme's active site (which involves cleavage of the sultone's S–O bond and formation of the nitrophenolate chromophore) and a subsequent slow desulfonylation to regenerate active enzyme and form the product acid III.[5]

The rate of the sulfonylation reaction is far too fast to be followed with a conventional spectrophotometer. However, a stopped-flow apparatus equipped with a spectrophotometer conveniently permits observation of the sulfonylation reaction and determination of the kinetic parameters.

The rather rapid hydroxide ion catalysis in the hydrolysis of II ($k_{OH} = 1 \times 10^3\ M^{-1}\ sec^{-1}$)[6] as well as catalysis by various buffer ions made determination of kinetic parameters with the sultone in excess impractical. This problem led to our decision to do the sulfonylation kinetics with the enzyme in excess. No corrections for "spontaneous" hydrolysis of II were found to be necessary under the conditions used for these experiments.

In the sulfonylation of CT by II, with a probable reaction sequence such as

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EP \qquad (II)$$

enzyme and sultone are kinetically equivalent. In the presence of a sufficient excess of enzyme one should observe spectrophotometrically a pseudo-first-order reaction with rate constant k related to the kinetic parameters by eq 1.

$$k = \frac{k_2 E}{(k_{-1} + k_2)/k_1 + E} = \frac{k_2 E}{K_s + E} \qquad (1)$$

Assume $k_{-1} >> k_2$, then K_s is defined to be k_{-1}/k_1. In all experiments first-order kinetics were observed. The reaction is found to be more than sufficiently fast to ignore the slow decomposition of EP to enzyme and product III. It was hoped that a complete pH–rate profile

of the pseudo-first-order rate constants at different enzyme concentrations would permit a determination of the pH dependence of k_2 and K_s separately by the use of double reciprocal plots at various intervals of pH. However, plots of $1/k$ on the ordinate against $1/E$ on the abscissa at several pH values gave no very conclusive evidence for a positive intercept on the $1/k$ axis at enzyme concentrations low enough so that dimerization of the enzyme did not become a possible problem.[7,8] Certainly, the accuracy necessary for a meaningful pH profile of the individual parameters could not be obtained. These observations led us to abandon any attempt to determine the pH dependence of the individual parameters and be satisfied instead with the pH profile of k_2/K_s. Admittedly, because of the difficulties we encountered we were unable to obtain direct kinetic evidence for the binding step in the reaction of sultone II with CT. As will be described shortly, however, it was possible to obtain kinetic evidence for the binding of the sultone I to CT, and by analogy it seems reasonable to postulate a binding step in the sulfonylation of CT by II.

It was also found that a smooth transition between phosphate and Tris buffers could not be obtained until they were adjusted to the same ionic strength. Stopped-flow kinetic studies on the acylation of CT by *p*-nitrophenyl acetate did not reveal this problem.[9] Therefore, a complete pH profile was obtained in buffers corrected to the same ionic strength and at low enough enzyme concentration so that, to a good approximation

$$k = (k_2/K_s)E \qquad (2)$$

The experimental results are plotted in Figure 1. A well-defined bell-shaped curve was obtained. The curve is a computer-calculated, least-squares fit, with $pK_1 = 7.044$ and $pK_2 = 8.677$.[10] A few experiments were made with sultone in excess to verify that the pseudo-first-order rate constants are the same as those obtained under conditions of excess enzyme. Although the spontaneous hydrolysis of sultone resulted in a larger experimental uncertainty, the results did indicate the equivalence of rate constants under the two different conditions at at least two pH values (see dotted points in Figure 1).

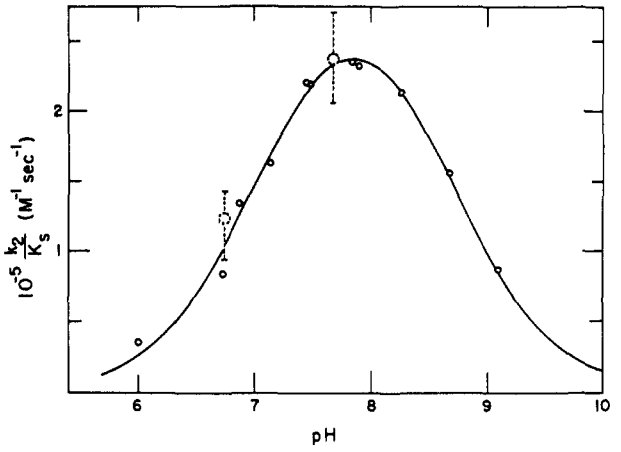


Figure 1. pH profile for the sulfonylation of α-chymotrypsin by sultone II at 25.0°. The curve is a theoretical one for $pK_1 = 7.044$, $pK_2 = 8.667$, and $k_2/k_s(\text{lim}) = 3.098 \times 10^5\ M^{-1}\ \text{sec}^{-1}$ (a computer-calculated, least-squares fit to the excess enzyme points). The solid circles represent points obtained with enzyme in excess and the dotted ones are for points found with sultone in excess. The solutions contained 0.02% CH_3CN, and the ionic strength was 0.2.

Inhibition Studies Using Stopped-Flow Spectrophotometry

If the sultones react at the normal active site of CT one would expect specific inhibitors of CT also to inhibit the reaction of CT with sultones. Since we are interested in obtaining kinetic evidence for the binding of sultones by CT we also considered using sultone I as an inhibitor for the reaction of CT with sultone II. Brief studies on the reaction of CT with I had revealed a presteady-state period of about 6 min, so we would expect no significant reaction of CT with I before the reaction with II was complete. There are no spectral complications at 3910 Å.

The specific inhibitor chosen was N-acetyl-L-tryptophanamide. No attempt was made to obtain inhibition constants from the ratio of slopes in double-reciprocal plots. Instead competitive inhibition was assumed, and inhibition constants were obtained from the ratio of rate constants in the presence and absence of inhibitors. Under conditions where $E << K_m$ the inhibition constant K_I is given by eq 3, where I is the

$$K_I = \frac{I}{(k/k') - 1} \qquad (3)$$

inhibitor concentration and k and k' are the pseudo-first-order rate constants in the absence and presence of inhibitor, respectively. The results of measurements on the inhibition by N-acetyl-L-tryptophanamide served as a check on the validity of the technique.

Two runs each were made for the sulfonylation of CT by II at pH 8 (a) in the absence of inhibitors, (b) in the presence of a given concentration of N-acetyl-L-tryptophanamide, and (c) in the presence of a nearly maximum possible concentration of sultone I. The experimental conditions and the observed rate constants are given in Table I.

Table I. Results of Stopped-Flow Inhibition Studies

Expt no.	Inhibitor	Inhibitor concn, M	k, sec^{-1}	k_{av}
1	None	0	12.2	
				12.6 ± 0.4
2	None	0	13.0	
3	N-Acetyl-L-tryptophanamide	8.01×10^{-3}	5.17	
				5.24 ± 0.08
4		8.01×10^{-3}	5.32	
5	2-Hydroxy-α-toluenesulfonic acid sultone	4.97×10^{-4}	9.46	
				9.39 ± 0.07
6		4.97×10^{-4}	9.32	

It can be seen that, without question, both compounds inhibit the sulfonylation reaction. Using eq 3 we obtain for N-acetyl-L-tryptophanamide a K_I of 5.7 ± 0.4 × 10^{-3} M. This value agrees well with K_I and K_s values obtained by other investigators.[11-13] For sul-

(7) Unpublished observations of J. H Heidema.
(8) F. J. Kezdy and M. L. Bender, *Biochemistry*, **4**, 104 (1965).
(9) *Cf.* M. L. Bender, G. E. Clement, F. J. Kezdy, and H. d'A Heck, *J. Am. Chem. Soc.*, **86**, 3680 (1964).
(10) A computer program written by Dr. P. L. Hall was used. See P. L. Hall, Ph.D. Thesis, University of Chicago, 1967.

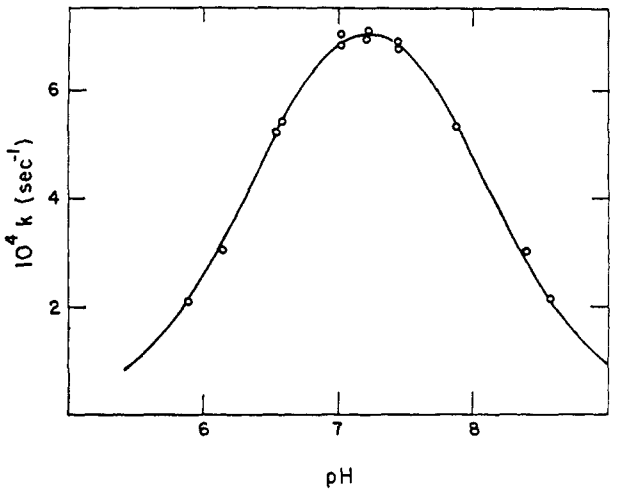
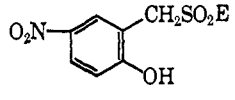


Figure 2. pH profile for desulfonylation of 2-hydroxy-5-nitro-α-toluenesulfonyl-α-chymotrypsin (IV) at 25.0°. The curve is a theoretical one for pK_1 = 6.408, pK_2 = 8.044, and k(lim) = 9.198 × 10^{-4} sec^{-1} (a computer-calculated, least-squares fit). The solutions contained 0.64% (v/v) CH_3CN.

tone I the data give $K_I = 1.4 \pm 0.2 \times 10^{-3}$ M, *i.e.*, it is a more effective inhibitor for this reaction than the specific inhibitor used. The value obtained is similar to the dissociation constant obtained for *p*-nitrophenyl acetate.[9]

Kinetics of the Desulfonylation Reaction

As we will elaborate on further in the discussion section, the presence of the *o*-hydroxyl group in the sulfonyl enzyme IV appears to be a structural requirement for the desulfonylation reaction to occur. In IV we would expect ionization of the phenolic proton with a pK_a value near 7.0, *i.e.*, right in the region where the enzyme is active. If the presence or absence of the



IV

phenolic proton is crucial, the pH dependence of the desulfonylation reaction should in some way reflect the pK_a of the nitrophenol.

We have made a kinetic investigation of the pH dependence of the rate of the first-order desulfonylation of IV. Sultone II was added to a molar excess of CT to produce the sulfonyl enzyme. Kinetic measurements were made by following the rate of increase in absorbance at 4110 Å after the initial "burst" in absorbance. The results are shown in Figure 2. The pH dependence is found to be bell-shaped. The curve of Figure 2 is a computer-calculated, least-squares fit to the data. The pK_a values thus determined are pK_1 = 6.41 and pK_2 = 8.04.[10] CT deacylations have been shown to exhibit a sigmoidal pH profile, one ionizing group of pK_a ~ 7.0 (presumably the imidazole ring of a histidine residue) being found necessary for decompositions of the

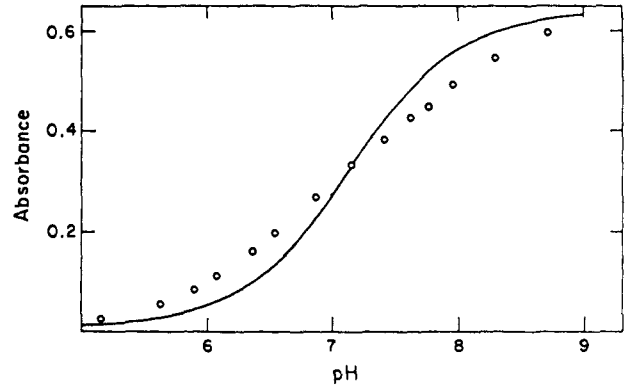
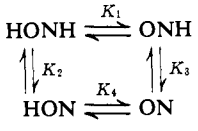


Figure 3. Absorbance of a given concentration of 2-hydroxy-5-nitro-α-toluenesulfonyl-α-chymotrypsin (IV) at 391 mμ *vs.* pH, in 0.05 M phosphate buffers containing 1.53% CH_3CN. Curve is a theoretical sigmoid for pK_a = 7.15 and A_{max} = 0.640, A_{min} = 0.010.

acyl enzymes.[14] It is possible that this group is also involved in the desulfonylation reaction with its pK_a in some way perturbed and that the nitrophenol ionization is the additional factor producing the bell-shaped pH profile. It should be possible to determine spectrophotometrically the pK_a of the nitrophenol group on the sulfonyl enzyme and compare this pK_a with those which have been determined kinetically. This has been done. We determined the pH dependence of the initial "burst" absorbance ΔA at 3910 Å upon adding a given amount of sultone II to an excess of CT. The data are plotted in Figure 3. The pH of half-maximum ΔA is approximately 7.15, far from the kinetically determined values. However, an examination of the spectral data reveals, as seen in Figure 3, that it does not conform to the shape of a theoretical sigmoid curve. By contrast, the data of a similar pH *vs.* absorbance profile for the product acid III very nicely fit a theoretical sigmoid for a pK_a of 7.10 (see Figure 4). These observations lead us to conclude that ionization of the phenolic proton on the sulfonyl enzyme is influenced by another ionization of a nearby group occurring at a similar pH.[15] A likely candidate for this group is the imidazole ring of a histidine residue,[16] perhaps the same group whose ionization is reflected in the desulfonylation pH profile. A scheme like III might therefore



(III)

be proposed as a reasonable explanation for both the kinetic and the spectral data, where HONH is the sulfonyl enzyme protonated at both the imidazole and the phenol, ON is the sulfonyl enzyme with both groups deprotonated, and ONH and HON are singly protonated species at the imidazole and phenol, respectively. The colored species would be ONH and ON. The species which would desulfonylate is tentatively HON. Such a scheme will give a bell-shaped pH profile, with velocity v defined by eq 4.

(11) Published values vary over the range 5.0–7.5 × 10^{-3} M. *Cf.* R. J. Foster and C. Niemann, *J. Am. Chem. Soc.*, 77, 1886 (1955), and ref 12 and 13.

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

(13) F. J. Kezdy, J. Feder, and M. L. Bender, *J. Am. Chem. Soc.*, **89**, 1009 (1967).

(14) M. L. Bender, G. L. Schonbaum, and B. Zerner, *ibid.*, **84**, 2562 (1962).

(15) For a study of a related phenomenon in aminothiol compounds see R. E. Benesch and R. Benesch, *ibid.*, 77, 5877 (1955).

(16) A preliminary X-ray structure determination of tosyl-CT places an imidazole in the vicinity of the modifier residue: B. W. Matthews, P. B. Sigler, R. Henderson, and D. M. Blow, *Nature*, **214**, 652 (1967).

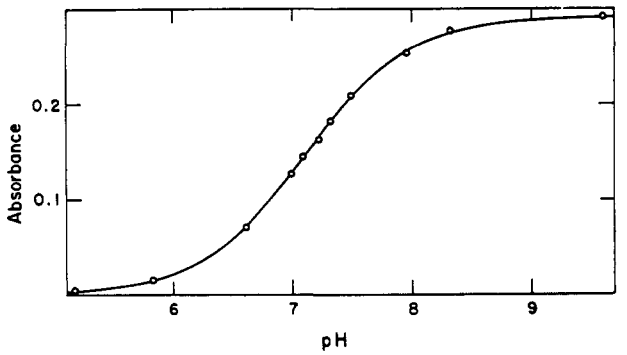


Figure 4. Absorbance of a given concentration of 2-hydroxy-5-nitro-α-toluenesulfonic acid at 411 mμ *vs.* pH in 0.05 *M* phosphate buffers containing 1.53% CH_3CN. Curve is a theoretical one for pK_a = 7.10 and maximum absorbance at 411 mμ = 0.292.

$$v = \frac{kT}{1 + K_1/K_2 + H/K_2 + K_4/H} = \frac{\frac{k}{1 + (K_1/K_2)}T}{1 + \{K_4/[1 + (K_1/K_2)]\}/H + H/(K_1 + K_2)} \quad (4)$$

Equation 4 has too many constants to define them all uniquely from the kinetic data. But a unique solution is possible if the spectral data are also employed. If the assumption is made that ONH and ON are the colored species and that both have the same extinction coefficients at 3910 Å, the following equations may be derived[17]

$$\frac{\Delta A}{\Delta A_\infty} = \frac{N/H + K_1/M}{1 + H/M + N/H} \quad (5)$$

$$\Delta A(1 + H/M + N/H) = \Delta A_\infty N(1/H) + \Delta A_\infty K_1 M \quad (6)$$

where

$$M = K_1 + K_2 \quad (7)$$

and

$$N = \frac{K_4}{1 + K_1/K_2} \quad (8)$$

and ΔA represents the absorbance at the hydrogen ion concentration H minus the absorbance at low pH and ΔA_∞ represents ΔA at high pH. M and N are simply the first and second ionization constants measured, respectively, in the desulfonylation kinetics.

These equations suggest two methods for determining the dissociation constants of mechanism III. One method would be to plot $\Delta A(1 + H/M + N/H)$ *vs.* $1/H$ using both the spectral and kinetic data. From the slope and intercept of the resulting straight line, K_1 could be determined from eq 6. The other constants would then be determined using eq 7 and 8 and the relationship $K_1K_3 = K_2K_4$. Such a plot has been made (see Figure 5). The calculated dissociation constants are given in Table II. A second method would be to use $\Delta A/\Delta A_\infty$ at one point on the experimental titration curve and the value of H at that point in eq 5 to determine K_1. A calculation of this nature has been made using the midpoint of the titration curve as pH 7.15. The results are given in Table II.

(17) See Mathematical Appendix.

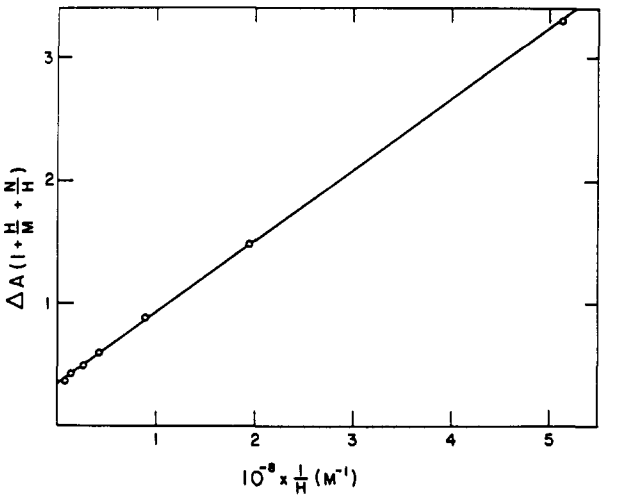


Figure 5. Data from the spectrophotometric titration curve of the sulfonyl enzyme IV plotted according to eq 6; intercept = 0.353, slope = 0.575 × 10^{-8} *M*.

Given the dissociation constants of eq III it should be possible to predict the shape of the experimental titration curve. The calculated curve for each of the two methods above is superimposed on the experimental data in Figures 6 and 7 (the slope-intercept method also predicts ΔA_∞ of 0.637; the curve of Figure 7 is based on the estimated ΔA_∞ of 0.630). These do not give perfect fits to the data, but they are certainly preferable in this regard to a single sigmoid. If reaction scheme III is correct, it may be that the imperfections in the calculated curves arise because the kinetic data are not sufficiently accurate to withstand this sensitive a test. Admittedly, there are numerous schemes which might fit our experimental results fairly well, and we obviously cannot claim that scheme III is a uniquely satisfactory one. However, we feel that it provides a reasonable explanation for our results, and at the present time there is no reason to postulate a more complex scheme.

Table II

	From slope and intercept using eq 6	From midpoint using eq 12
K_1, pK_1	2.169 × 10^{-7} *M*, 6.664	2.061 × 10^{-7} *M*, 6.686
K_2, pK_2	1.745 × 10^{-7} *M*, 6.758	1.852 × 10^{-7} *M*, 6.732
K_3, pK_3	1.629 × 10^{-8} *M*, 7.788	1.714 × 10^{-8} *M*, 7.766
K_4, pK_4	2.025 × 10^{-8} *M*, 7.694	1.908 × 10^{-8} *M*, 7.719

Reaction of CT with 2-Hydroxy-3,5-dinitro-α-toluenesulfonic Acid Sultone (V)

One feature of the desulfonylation mechanism suggested by the kinetic studies is that the *o*-hydroxyl group of the sulfonyl enzyme IV must be protonated for desulfonylation to occur. These observations led us to test the possibility that a similar sulfonyl enzyme with a more acidic *o*-hydroxyl group will not desulfonylate at pH values near neutrality. If the dinitro-substituted sultone V could be used to sulfonylate CT, according to the above hypothesis, the resulting sulfonyl enzyme VI should be stable at pH values near 7 since the *o*-hy-

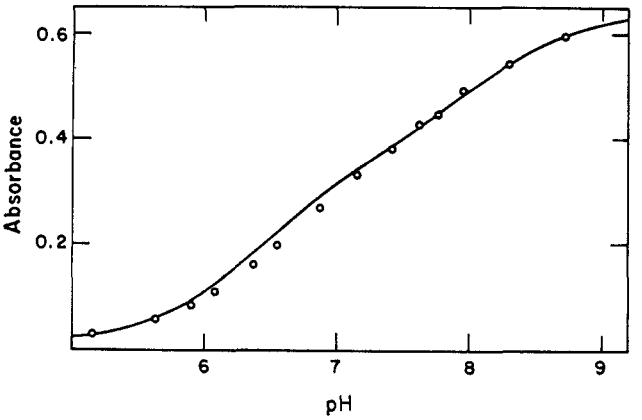


Figure 6. Data of Figure 3. Curve is a calculated one based on reaction scheme III with equilibrium constants and ΔA_∞ as determined from the slope and intercept of Figure 5.

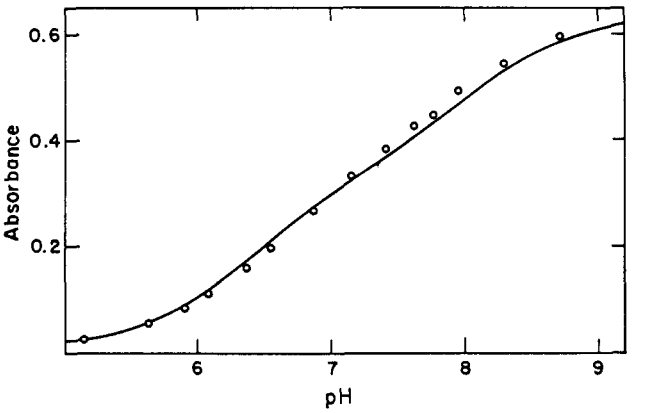
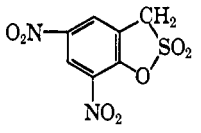
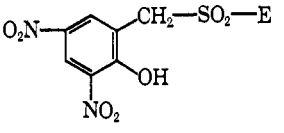


Figure 7. Data of Figure 3. Curve is a calculated one based on reaction scheme III with equilibrium constants as determined using eq 12. A_∞ estimated as 0.630.

droxyl group would be essentially deprotonated down to about pH 5.



V



VI

The base-catalyzed hydrolysis of compound V is extremely rapid so that its addition to a CT solution will not effect modification unless very efficient mixing is achieved during the addition. CT solutions were treated with an acetonitrile solution of V and with pure acetonitrile using identical procedures. The solutions in pH 7.2 buffer, were rate assayed at various time intervals with the substrate N-carbobenzyloxy-L-tyrosine *p*-nitrophenyl ester. The results are shown in Table III.

Table III. Results of the Sulfonylation of CT by Sultone V

Sample	Time, min after addition of sultone	Relative activity,[a] $10^6 \times$ OD units/sec
CT + CH_3CN	8	279
	223	242
CT + sultone V in CH_3CN	7	26
	103	21
	267	19

[a] Using N-carbobenzyloxyl-L-tyrosine *p*-nitrophenyl ester as substrate at 3210 Å; pH 5.20 (acetate); 25.4°.

As can be seen, the procedure effected approximately 90% inhibition, and no tendency of the inhibited enzyme to reactivate was noticeable over 4.5 hr. The data indicate that, as was anticipated, the sulfonyl enzyme VI formed from sultone V does not desulfonylate at neutral pH value.

Experimental Section

Materials. α-Chymotrypsin, three times recrystallized and lyophilized, was obtained from three sources. Preliminary studies and measurements on the desulfonylation kinetics were done with several preparations purchased from the Sigma Chemical Company. Stopped-flow kinetics and the work with the dinitro-substituted sultone, V were done with enzymes from Worthington Biochemical Corp. (Lot. No. CDI 61J), except for the inhibition studies for which material received as a gift from the Armour Pharmaceutical Co. was used.[18] Except where indicated stock solutions of enzyme were prepared by dissolving a given weight of dry enzyme into pH 5.05 acetate buffer (0.15 *M* total acetate) and were kept refrigerated at 4°. Enzyme solutions were routinely titrated with N-*trans*-cinnamoylimidazole by the method of Schonbaum, *et al.*,[19] at 3350 Å.

2-Hydroxy-α-toluenesulfonic acid sultone (I) was a gift from Mr. O. R. Zaborsky.[3] The 5-nitro and 3,5-dinitro derivatives II and V were a gift from Mr. K. W. Lo.[6] The structures of all three sultones have been confirmed by various instrumental methods as well as by elemental analysis. Sultone I and the nitro-substituted sultone II were recrystallized from dry ethanol before use. The dinitro derivative V was recrystallized from acetone–cyclohexane (mp 225–226°). N-*trans*-Cinnamoylimidazole was made according to the procedure of Schonbaum, *et al.*,[19] mp 132.5–133.4°. N-Acetyl-L-tryptophanamide was a product of Mann Research Laboratories (mp 192–194°) and was used without further purification. N-Carbobenzyloxy-L-tyrosine *p*-nitrophenyl ester was purchased from Mann Research Laboratories and recrystallized twice from chloroform–hexane, mp 157–158°.[20]

Solutions of all three sultones and the N-*trans*-cinnamoylimidazole were made up in reagent grade acetonitrile from the J. T. Baker Chemical Co. No effect whatsoever on spectrophotometric absorbance readings was observed at the wavelengths and acetonitrile concentrations used in this work when reagent grade material was used. Although this acetonitrile has a small amount of water in it (<0.05%) even the dinitrosultone solutions are stable for several months. The use of spectral grade material for this kind of work would therefore seem unnecessary. All inorganic compounds used in making buffer solutions were of reagent grade. 2-Amino-2-hydroxymethyl-1,3-propanediol (Tris) was purchased from Matheson Coleman and Bell (highest purity, mp 170–171°). 2-Amino-2-methyl-1,3-propanediol (Ammediol) was purchased from Matheson Coleman and Bell, and was recrystallized from ethyl acetate, mp 109.6–111.1°. All water used in this work was distilled and then passed through a mixed-bed, ion-exchange column (Continental Demineralization Service).

General Procedural Techniques Used in Preliminary Studies. The spectral studies were performed on a Cary 14 recording spectrophotometer. Reactions were carried out in quartz cuvettes of ~4 ml total volume. The enzyme and other reagents were added by λ pipet from stock solutions to buffered solutions of water (usually 3.00 ml). No reference cell was usually used unless it was desired to "blank out" a given absorbance. When the exact time of addition of a reagent was desired, an electric timer with a push-button starter reading to tenths of a second was employed.

pK_a Determinations. A solution of 2-hydroxy-5-nitro-α-toluenesulfonic acid[21] was prepared in 0.05 *M* Na_2HPO_4. To 3.00 ml of 0.05 *M* phosphate buffer at the desired pH + 50 μl of acetonitrile was added 250 μl of the solution of sulfonic acid. The increase in absorbance at 3210 and 4110 Å upon addition of the acid was meas-

(18) We wish to thank Dr. J. P. Dailey for this generous gift.
(19) G. R. Schonbaum, B. Zerner, and M. L. Bender, *J. Biol. Chem.*, **236**, 2930 (1961).
(20) We thank Professor F. J. Kezdy for a sample of this compound.
(21) This compound was a gift from K. W. Lo.

ured on a Cary 14. The pH was then measured on a Radiometer pH meter, type PHM 4c, standardized before each determination against a fresh pH 7.40 standard buffer solution (Fisher Certified) at 25°. The same three pipets were used for each determination. A similar procedure was used to determine the pK_a of the nitrophenolic group in the sulfonyl enzyme IV. To 3.00 ml of 0.05 *M* phosphate buffer at the pH desired plus 250 μl of 1.0×10^{-3} *M* CT in pH 7.0 phosphate buffer (0.05 *M*) was added (with subsequent stirring) 50 μl of 3.07×10^{-3} *M* nitrosultone in acetonitrile. The excess of active enzyme results in all the sultone being converted to sulfonyl enzyme. The absorbance "burst" at 3910 Å was measured on a Cary 14. For one determination (at the lowest pH) 0.15 *M* acetate buffer was used. All pH determinations were made as above within ~20 min of sultone addition. The same three pipets were used in each determination (and, of course, were washed before the next determination).

The Reaction of CT with 2-Hydroxy-3,5-dinitro-α-toluenesulfonic Acid Sultone (V). To 3.00 ml of 0.10 *M* acetate buffer at pH 6.0 and 200 μl of 5.4×10^{-4} *M* CT in 0.15 *M* acetate buffer at pH 5.05 was added at room temperature with vigorous magnetic stirring three successive portions of 25 μl each of a 3.0×10^{-3} *M* solution of the dinitrosultone V in acetonitrile (over a period of ~90 sec). This was designated solution A. To 5.00 ml of 0.05 *M* phosphate buffer at pH 7.24 was added 100 μl of solution A. This solution (designated solution B) was stored in the thermostated cell compartment of a Cary 14 at 25.0 ± 0.5°. The procedure was duplicated using pure acetonitrile in place of the dinitrosultone solution. The experimentally determined pH of solution A was 5.83 and that of solution B was 7.21. At appropriate time intervals a portion of solution B was rate assayed with N-carbobenzyloxy-L-tyrosine *p*-nitrophenyl ester (CTN) as substrate by the following procedure. CTN, 50 μl of 9.6×10^{-4} *M*, in acetonitrile was added to 3.00 ml. of pH 5.20 acetate buffer (0.10 *M*). The solution was thermostated in the cell holder of a Cary 14 spectrophotometer for at least 15 min (temperature of all assays was 25.4 ± 0.1°). To this solution was then added 100 μl of solution B. The absorbance increase at 3210 Å was measured *vs.* time (proportional to the rate of formation of *p*-nitrophenol) using a 0.0–0.2 absorbance unit slide wire.

Kinetic Measurements. Temperature readings reported here were determined with thermometers calibrated by a standard thermometer which itself was calibrated at the National Bureau of Standards. pH measurements were made on Beckman Research pH meters standardized against fresh Fisher Certified standard buffers. In the stopped-flow studies the standard 7.40 buffer was compared to a Bates primary standard buffer of pH 7.413 at 25.0°.[22]

In the desulfonylation pH-profile experiments a typical kinetic run was made according to the following procedure. To 3.00 ml of 0.05 *M* phosphate buffer at the desired pH was added 100 μl of a 1.0×10^{-3} *M* solution of CT in 0.05 *M* phosphate buffer at pH 7.5. After allowing time for the solution to attain 25.0 ± 0.1° in the thermostated cell compartment of the Cary 14 spectrophotometer, 20 μl of a 3.07×10^{-3} *M* solution of the nitrosultone V in acetonitrile (or 10 μl of nitrosultone solution plus 10 μl of acetonitrile if the size of the absorbance change required it) was added, the solution was stirred, the cuvette was capped with a ground-glass stopper, and the increase in absorbance at 4110 Å was recorded *vs.* time. The spectrophotometer was equipped with a 0.0–0.2 absorbance unit slide wire for all determinations. At pH values below 6.5 the absorbance increase at 3210 Å was found to be more useful. The pH of the solution was determined after the run was completed. The pH change during the course of a kinetic run at the extreme pH values was found to be only about −0.01 units. The temperature of a solution in the cell holder was determined before and after each day's kinetic runs. A time lapse of ~2.5 months occurred between the majority of runs and those performed at pH 5.89, 6.14, and 6.58. As can be seen the overlap with data obtained previously is quite satisfactory.

The kinetic studies of the sulfonylation reaction were performed on a Durrum-Gibson stopped-flow spectrophotometer, a commercial instrument available from the Durrum Instrument Corp., Palo Alto, Calif. The sultone solutions were prepared shortly before use by diluting a stock solution of 1.008×10^{-2} *M* nitrosultone V in acetonitrile with enough distilled and deionized water (plus acetonitrile as necessary to maintain the same concentration of organic solvent) to give the desired concentration. For the runs with excess substrate a 10^{-5} *M* HCl solution was used in diluting the acetonitrile solution of the nitrosultone. Inhibitors in the inhibition study were dissolved in the nitrosultone solution, not in the enzyme solution. Enzyme solutions were prepared less than 1 hr before use. Dry enzyme of the desired weight was transferred to a 10-ml volumetric flask. The flasks were then stoppered and stored at refrigerator temperatures until shortly before use when buffer at the pH and at twice the concentration desired was added to give 10.00 ml of solution. Enzyme concentrations were determined by dissolving a known dry weight of the particular enzyme batch into pH 5.05 acetate buffer (0.15 *M*), diluting to 10.00 ml, and titrating the resulting solution with N-*trans*-cinnamoylimidazole.

Since a single determination required only 0.2 ml of each of the two solutions, several trial runs could be made. Experimental traces were retained on the screen of the storage oscilloscope and were not usually photographed unless two successive runs gave superimposable traces. When this was not possible traces of at least two different runs were photographed. Time was allowed before recorded runs were made for the two solutions to come to the temperature of the constant temperature bath (25.0 ± 0.1°). pH measurements on the reaction solution were made immediately after the runs were recorded.

The oscilloscope records linearly in per cent transmittance with any desired transmittance as a base line and with a full-scale deflection suitable for the change in transmittance desired. The calculations required to convert the points of an oscilloscope trace to a table of absorbances at given time intervals were usually programmed into a Monroe Epic 3000 desk computer.

Conclusions

The kinetics of the sulfonylation of CT by the nitro-substituted sultone II revealed a bell-shaped pH dependence for the function k_2/K_S with p*K* values of 7.04 and 8.67 for the groups on the enzyme responsible for this behavior. Similar results have been obtained for a number of CT carboxylic ester substrates.[9] The group with a p*K* near 7.0 is presumably an imidazole ring of a histidine residue which is catalytically active only in its unprotonated form. The ionization with a p*K* which generally appears to fall between 8.6 and 9.0 has been attributed in recent papers to a group affecting binding, possibly an N-terminal isoleucine.[12,23,24]

Previous studies with several substrates indicate that the functions k_2 and K_S both vary sigmoidally with pH.[9] The nonspecific substrate *p*-nitrophenyl acetate appears to show exceptional behavior in that k_2 shows a bell-shaped profile while K_S is essentially pH independent.[9] The results given in the present paper are not sufficient to determine which of the above two types of behavior is characteristic of our sultone substrates.

It is well known that CT and other serine proteinases are inhibited by various phosphoryl and sulfonyl halides. The inhibitor most similar to the cyclic sulfonates investigated here is α-toluenesulfonyl fluoride. This compound reacts stoichiometrically with CT to produce an enzyme molecule sulfonylated at the active-site serine hydroxyl.[25] It has been shown that there is no detectable desulfonylation of α-toluenesulfonyl-α-chymotrypsin at 25° in the pH range 3–9.[26] It is highly probably that the site of the sulfonylation by sultones is the active-site serine hydroxyl also although degradative evidence on this point is lacking.[27] If we indicate

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(23) A. Himoe, P. C. Parks, and G. P. Hess, *J. Biol. Chem.*, **242**, 919 (1967).

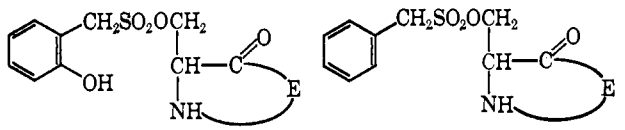
(24) M. L. Bender and F. C. Wedler, *J. Am. Chem. Soc.*, **89**, 3052 (1967).

(25) (a) A. M. Gold and D. E. Fahrney, *Biochemistry*, **3**, 783 (1964); (b) A. M. Gold, *ibid.*, **4**, 897 (1965); (c) J. Kallos and D. Rizok, *J. Mol. Biol.*, **9**, 255 (1964).

(26) D. E. Fahrney, Ph.D. Thesis, Columbia University, 1963.

(27) We have demonstrated stoichiometric attachment of the sulfonyl group to the active site. All of the many other reagents which behave similarly and cause complete inhibition of chymotryptic activity have been shown to be attached either to serine-195 or histidine-57 (on the

the structures of the sulfonyl enzymes resulting from the reaction of CT with sultone I and α-toluenesulfonyl fluoride by VII and VIII, respectively, it is apparent that the only structural difference between the two sulfonyl enzymes is the presence of the *o*-hydroxyl group in VII. From our work on the question of the desulfonylation of the sulfonylchymotrypsins formed from the reactions of sultones I, II, and V, the *o*-hydroxyl group clearly must be participating catalytically only in its protonated form. At the present time the mechanistic role of an *o*-phenolic hydroxyl in the desulfonylation of sulfonylchymotrypsins is unclear.[28] Among numerous possibilities, the phenolic group might be acting as a general acid catalyst for desulfonylation or it could be inducing a conformational change needed for desulfonylation to occur. In any event, it is obvious that further work on the role of the phenolic hydroxyl must be done, and studies along these lines are in progress in our laboratory.



VII

VIII

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Mathematical Appendix

Consider reaction scheme III for the desulfonylation of the sulfonyl enzyme IV. We assume that the two colored species are ONH and ON and that the only catalytically active species is HONH. Suppose that $\Delta\epsilon_1$ represents the difference at 391 mμ between the extinction coefficient of ONH and those of the species which are protonated at the oxygen and that $\Delta\epsilon_2$ represents the corresponding difference for ON. Let T = the total concentration of species (HONH) + (HON) + (ONH) + (ON). $\Delta A_\infty = \Delta\epsilon_2 lT$ where l = the path length.

$$\frac{\Delta A}{\Delta A_\infty} = \frac{\Delta\epsilon_1(\mathrm{ONH}) + \Delta\epsilon_2(\mathrm{ON})}{\Delta\epsilon_2 T} = \frac{(\Delta\epsilon_1/\Delta\epsilon_2)(\mathrm{ONH}) + (\mathrm{ON})}{T} \quad (9)$$

From reaction scheme III it follows that

$$\frac{\Delta A}{\Delta A_\infty} = \frac{(\Delta\epsilon_1/\Delta\epsilon_2)(K_1/K_2)(\mathrm{HON}) + (K_4/H)(\mathrm{HON})}{T}$$

$$= \frac{\left[\frac{K_4}{1 + (K_1/K_2)}\right]\left(\frac{1}{H}\right) + (\Delta\epsilon_1/\Delta\epsilon_2)[K_1/(K_1 + K_2)]}{1 + [H/(K_1 + K_2)] + \left[\frac{K_4}{1 + (K_1/K_2)}\right]\left(\frac{1}{H}\right)}$$

$$= \frac{N/H + (\Delta\epsilon_1/\Delta\epsilon_2)(K_1/M)}{1 + H/M + N/H} \quad (10)$$

where

$$M = K_1 + K_2 \quad (7)$$

$$N = K_4/[1 + (K_1/K_2)] \quad (8)$$

and M and N can be obtained from the kinetic data.

$$\Delta A(1 + H/M + N/H) = \Delta A_\infty N(1/H) + (\Delta\epsilon/\Delta\epsilon_2)\Delta A_\infty(K_1/M) \quad (11)$$

Now if $\Delta A(1 + H/M + N/H)$ is plotted against $(1/H)$ a straight line should be obtained with slope = $\Delta A_\infty N$ and intercept = $(\Delta\epsilon_1/\Delta\epsilon_2)\ \Delta A_\infty(K_1/M)$. A unique value for K_1 can be obtained if a value for the ratio $(\Delta\epsilon_1/\Delta\epsilon_2)$ is assumed. In the absence of any firm information about this ratio we assume that $(\Delta\epsilon_1/\Delta\epsilon_2) = 1$. Although we are aware that the two extinction coefficients $\Delta\epsilon_1$ and $\Delta\epsilon_2$ may not be identical the assumption we have made is probably the best we can do. With this assumption eq 10 reduces to eq 5 which was given earlier in this paper. Since K_1 can be obtained thus, and M and N are experimentally determined parameters, values for K_2, K_3, and K_4 can be calculated.

An alternative approach is to use $\Delta A/\Delta A_\infty$ at one point in the experimental spectrophotometric titration curve and the value of (H) in eq 10 to determine K_1. For instance, the following equation holds for the midpoint (H_m) of the titration curve if the ratio $\Delta\epsilon_1/\Delta\epsilon_2$ is taken as unity. As has been discussed earlier in this manuscript both this method and the method described in the paragraph above have been employed to calculate the ionization constants of reaction scheme III.

$$K_1 = \left[\frac{1}{2}\left(1 + \frac{H_m}{M} + \frac{N}{H_m}\right) - \frac{N}{H_m}\right]M \quad (12)$$

imidazole ring). Evidence obtained by many other investigators indicates that all acyl-, phosphoryl-, and sulfonylchymotrypsins previously investigated are modified at serine-195. Some recent pertinent reviews are: T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," W. A. Benjamin, Inc., New York, N. Y., 1966, pp 228–242; M. L. Bender and F. J. Kezdy, *Ann. Rev. Biochem.*, **34**, 49 (1965).

(28) We can presumably eliminate nucleophilic attack at sulfur by the phenolic oxygen leading to release of the original sultone and subsequent solvent-catalyzed hydrolysis since this pathway is inconsistent with our observations that the hydrolysis of the unsubstituted sultone I is *accelerated* by chymotrypsin. The CT-catalyzed hydrolysis of I was only briefly investigated. See ref 5.