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Enzymatic and Nonenzymatic Reactions of Cyclic Sulfonate and Sulfate Esters

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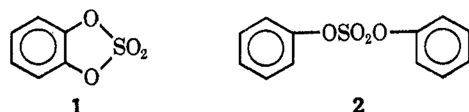
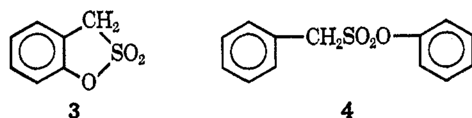
Several years ago we discovered that the five-membered cyclic sulfate ester, catechol cyclic sulfate (**1**), hydrolyzes in an alkaline medium more than 10^7 times faster than does the corresponding acyclic analog diphenyl sulfate (**2**).[1] After our observations on the

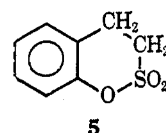


hydrolytic reactivity of catechol cyclic sulfate we turned to the corresponding cyclic sulfonate system. When the rate constant for the hydroxide ion catalyzed hydrolysis of 2-hydroxy-α-toluenesulfonic acid sultone (**3**) was measured it was found to be almost 10^6 times greater than the rate constant for the hydrolysis of the open-chain ester phenyl α-toluenesulfonate (**4**).[2]



Having observed the large rate accelerations for the hydrolyses of the five-membered cyclic sulfates and sulfonates, we explored the question whether the lability of these esters was confined to this particular ring size. The six-membered cyclic sulfonate **5** was prepared and the rate of its alkaline hydrolysis determined. Although the rate constant for the alkaline hydrolysis of **5** was somewhat larger than that for the reaction of the open-chain compound phenyl α-toluenesulfonate it was much smaller than that for the five-membered cyclic ester **3**.[3] Thus, it appears that the five-membered cyclic esters are uniquely labile.

Since the discovery of the exceptional reactivity of the five-membered cyclic sulfonate and sulfate esters,



we have devoted considerable attention to their reactions under enzymatic and nonenzymatic conditions. In the present review we will discuss the historical background and the current status of our investigations concerning the pathways by which the labile five-membered cyclic esters react with enzymes and other catalysts.

Historical Background

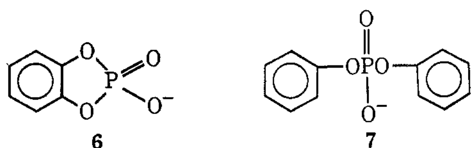
Prior to our studies enormous enhancement in hydrolytic lability had been observed in the case of five-membered cyclic phosphate esters,[4] but our observations with the cyclic sulfate and sulfonate esters were the first instances in which this kind of phenomenon had been seen outside of the phosphorus series. The five-membered cyclic ester ethylene phosphate hydrolyzes exclusively with P–O bond cleavage in alkaline solution, whereas the open-chain analog, dimethyl phosphate, reacts primarily by way of C–O cleavage.[5,6] When this difference in the modes of bond cleavage is taken into account, the rate enhancement for the attack at the phosphorus atom of the cyclic ester by hydroxide ion can be estimated to be greater than 10^8.

The initial attempts to find examples of reactivity comparable to the five-membered cyclic phosphates in another series were carried out with the substrates ethylene sulfate, trimethylene sulfate, and dimethyl sulfate.[7] It was found that the second-order rate constant for the alkaline hydrolysis of ethylene sulfate, the

(1) E. T. Kaiser, I. R. Katz, and T. F. Wulfers, *J. Amer. Chem. Soc.*, **87**, 3781 (1965).
(2) O. R. Zaborsky and E. T. Kaiser, *ibid.*, **88**, 3084 (1966).
(3) E. T. Kaiser and O. R. Zaborsky, *ibid.*, **89**, 1393 (1967).
(4) A review on the cyclic phosphate esters has appeared: F. H. Westheimer, *Accounts Chem. Res.*, **1**, 70 (1968).
(5) P. C. Haake and F. H. Westheimer, *J. Amer. Chem. Soc.*, **83**, 1102 (1961).
(6) C. A. Bunton, D. R. Llewellyn, K. G. Oldham, and C. A. Vernon, *J. Chem. Soc.*, 3574 (1958).
(7) E. T. Kaiser, M. Panar, and F. H. Westheimer, *J. Amer. Chem. Soc.*, **85**, 602 (1963).

five-membered cyclic species, was only 20 times greater than that for dimethyl sulfate and 100 times greater than that for trimethylene sulfate at 25°. However, the rate enhancement for attack at the sulfur atom in the five-membered cyclic ester could not be measured since ethylene sulfate reacts with only about 14% S–O bond cleavage, and within the limits of experimental detection dimethyl sulfate and trimethylene sulfate hydrolyze exclusively with C–O cleavage.

The rationale for our comparative studies on the hydrolysis of catechol cyclic sulfate (**1**) and diphenyl sulfate (**2**) which led to the results mentioned in the introduction was that nucleophilic attack of hydroxide ion at the aromatic carbon atoms in these compounds would be very unlikely. Hence, the difference in the rates of hydrolysis of the two esters would represent the difference in the rate of attack of hydroxide ion at sulfur for a five-membered cyclic sulfate compared to that for its open-chain analog. As already discussed, in contrast to the situation found with the five-membered cyclic aliphatic sulfate (ethylene sulfate), the aromatic sulfate (catechol cyclic sulfate) was very reactive compared to its acyclic analog. In view of this difference between the aliphatic and aromatic five-membered cyclic sulfate, we were interested in determining what the lability of a five-membered cyclic aromatic phosphate was relative to its acyclic analog. The five-membered cyclic aromatic phosphate, catechol cyclic phosphate (**6**), undergoes alkaline hydrolysis nearly 10^7 times faster than its open-chain analog, diphenyl phosphate (**7**), does.[8]



This rate acceleration is comparable to that observed for the hydrolysis of the aliphatic five-membered cyclic phosphate, ethylene phosphate.[4,5]

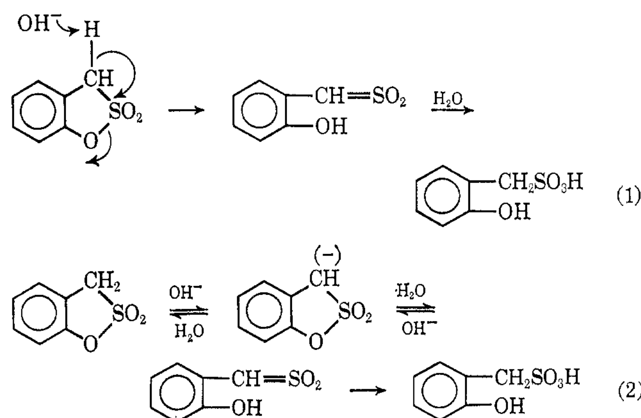
Position of Bond Cleavage in the Hydrolysis of Catechol Cyclic Sulfate

Our hypothesis that attack by hydroxide ion on catechol cyclic sulfate (**1**) should occur at the sulfur atom rather than at the aromatic carbon atoms was subjected to experimental test by the use of ^{18}O-labeled solvent. When **1** was hydrolyzed in alkaline ^{18}O-enriched solvent and then catechol monosulfate (**8**) which was produced was hydrolyzed further in an acidic ^{18}O-enriched solution, the catechol isolated was found to be devoid of excess oxygen-18. This result indicated that neither in the hydroxide ion catalysis of the cyclic sulfate **1** to the monoester **8** nor in the further acidic hydrolysis of **8** to form catechol and inorganic sulfate was aromatic carbon–oxygen fission taking place.[9]

From the oxygen-18 experiments it can be concluded that the rate acceleration observed for the hydrolysis of catechol cyclic sulfate relative to diphenyl sulfate does indeed reflect the difference in the rates of attack of hydroxide ion at the sulfur atoms in a five-membered cyclic sulfate and an open-chain sulfate, respectively. However, in the case of the cyclic sulfonate 2-hydroxy-α-toluenesulfonic acid sultone (**3**), hydrolytic mechanisms which do not involve direct attack of hydroxide ion at sulfur must be considered.

The Possible Formation of Carbanion and/or Sulfene Intermediates in the Hydrolysis of 2-Hydroxy-α-toluenesulfonic Acid Sultone

Specifically, the mechanisms outlined in eq 1 and 2 where carbanions and/or sulfenes may be reactive intermediates in the hydrolysis of the cyclic sulfonate **3** can be proposed. These mechanisms have been subjected to a variety of experimental tests. A prediction which could be made if the concerted elimination reaction shown in eq 1 occurs is that, if the reaction were run in D_2O, the resultant sulfonic acid should have one deuterium atom in the methylene group. The hydrolytic reaction can be studied under conditions where the hydrogens of the methylene group of the product sulfonic acid do not exchange with the deuterium of the solvent D_2O. When the hydrolysis of compound **3** was conducted in a D_2O–OD^- solution in which the sultone was in excess over OD^-, and **3** could be recovered after all the OD^- was consumed, the sultone **3** was found to have undergone extensive exchange of deuterium into the methylene group. From this observation it is clear that a carbanion is formed rapidly and reversibly from the sultone **3** in basic solution, and it is not a simple matter to decide whether carbanion and/or sulfene intermediates lie along the reaction pathway for the alkaline hydrolysis of **3**.

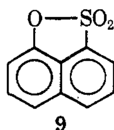


An experimental approach involving the measurement of the solvent isotope effect for the decomposition of the fully ionized ester was used to determine whether a mechanism analogous to that given in eq 2 applies to the alkaline hydrolysis of 5-nitrocoumaranone, a lactone with labile α protons which undergo ionization with a pK_a of 9.8.[10] This approach is limited to cases in which

(8) E. T. Kaiser and K. Kudo, *J. Amer. Chem. Soc.*, **89**, 6725 (1967).
(9) E. T. Kaiser and O. R. Zaborsky, *ibid.*, **90**, 4626 (1968).
(10) P. S. Tobias and F. J. Kézdy, *ibid.*, **91**, 5171 (1969).

the carbon acid is fully ionized at an alkalinity accessible in aqueous media. The pK_a for the ionization of the labile α protons of the sulfonate ester **3** is greater than 14. Therefore, we found it necessary to develop several general methods involving comparative isotope exchange and hydrolysis rate measurements to determine whether or not carbanions are intermediates in the alkaline hydrolysis of an ester.[11] On the basis of results obtained with these methods we now believe that the large rate enhancement we have observed for the alkaline hydrolysis of the cyclic ester **3** relative to its open-chain analog reflects the difference in the rate of attack of hydroxide ion at the sulfur atoms in the cyclic and acyclic systems. In other words, mechanisms involving carbanion and/or sulfene intermediates do not appear to provide the predominant pathways by which the five-membered cyclic sulfonate **3** hydrolyzes.

In an earlier study we showed that 1-naphthol-8-sulfonic acid sultone (**9**) hydrolyzes in alkali at a rate comparable to that for **3**.[3] Since **9** does not have a methylene group it cannot be reacting by way of carbanion and/or sulfene intermediates. Although our observation that **9** is very labile to alkali does not bear upon the occurrence or nonoccurrence of such intermediates in the hydrolysis of 2-hydroxy-α-toluenesulfonic acid sultone (**3**), it demonstrates at the very least that a sulfene pathway is not obligatory for the rapid hydrolysis of a five-membered sultone.



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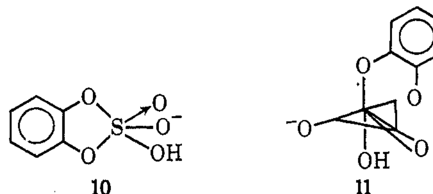
Pentacoordinate Intermediates in the Hydrolyses of Cyclic Sulfate and Sulfonate Esters

A question which we have explored in the hydrolyses of the cyclic sulfate and sulfonate esters is whether during these solvolyses there might be reversible formation of pentacoordinate intermediates in which the attacking hydroxide ion is covalently bound to sulfur.[9] This question was explored with the use of ^{18}O-enriched solvent. When incomplete hydrolyses of catechol cyclic sulfate (**1**), 2-hydroxy-α-toluenesulfonic acid sultone (**3**), and β-2-hydroxyphenylethanesulfonic acid sultone (**5**) were carried out in alkaline solutions containing excess oxygen-18 no significant exchange was observed when we reisolated the unconverted starting esters. These results show that in the hydrolysis reactions there is no detectable reversible formation of pentacoordinate intermediates such as **10** in which the oxygens attached to the sulfur atom and external to the ring have become equilibrated. Of course, our data do not rule out the possibility that pentacoordinate intermediates are formed irreversibly. Alternatively, pentacoordinate intermediates might be reversibly formed in the hydrolyses of the cyclic esters, and the oxygens external to the ring might not equilibrate during the lifetimes of these intermediates.[12]



10 11

It is an interesting possibility that if a pentacoordinate intermediate **10** is formed in the alkaline hydrolysis of catechol cyclic sulfate, for instance, and if the intermediate has a trigonal-bipyramidal geometry (see structure **11**), the reason why the oxygens external to the ring do not equilibrate might be that pseudorotation is much less facile than in the corresponding phosphorus systems.[4]

Structures of Five-Membered Cyclic Sulfate and Sulfonate Esters

The main driving force for the rapid hydrolysis of the five-membered cyclic sulfate and sulfonate esters is probably ring strain. The heat of hydrolysis of ethylene sulfate exceeds that of dimethyl sulfate by 5–6 kcal/mol.[7] The structures of the five-membered cyclic sulfates ethylene sulfate,[13a] vinylene sulfate,[13a] and catechol cyclic sulfate[13b] (**1**) and the cyclic sulfonate 2-hydroxy-α-toluenesulfonic acid sultone[13c] (**3**) have been determined by X-ray crystallography. One feature common to all of these structures is the small value of the ring angle around the sulfur atom which reflects the strain present in the five-membered rings. The internal O–S–O bond angle is 98.4° in ethylene sulfate, 93.6° in vinylene sulfate, and 97.1 in **1**, and the corresponding C–S–O bond angle in **3** is 96.1°. As discussed in the previous section there is no compelling evidence for the postulation of pentacoordinate intermediates in the alkaline hydrolyses of the five-membered cyclic esters. However, the transition states in these hydrolytic reactions may have structures with approximately trigonal-bipyramidal geometry in which the ring angle at sulfur is close to 90° and the five-membered ring spans one apical and one equatorial position. Relatively little perturbation of the ring angle at sulfur in the five-membered cyclic esters would be required to achieve such a transition-state geometry.

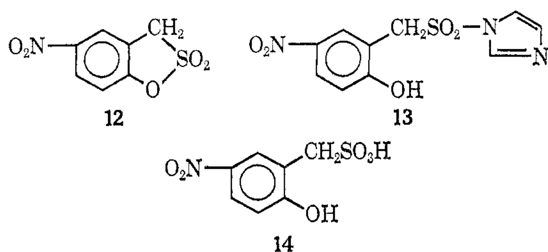
(11) For a discussion of the methods employed, the reader is referred to P. Müller, D. F. Mayers, O. R. Zaborsky, and E. T. Kaiser, *J. Amer. Chem. Soc.*, **91**, 6732 (1969).

(12) "Pentacoordinate" sulfur compounds were postulated as intermediates in organic reactions in a paper by B. M. Trost, R. LaRochelle, and R. C. Atkins, *J. Amer. Chem. Soc.*, **91**, 2975 (1969). Also, a test for pseudorotation in "pentacoordinate" sulfur compounds has been described by R. Tang and K. Mislow, *ibid.*, **91**, 5644 (1969). However, in these instances, the term "pentacoordinate" was employed to describe sulfur compounds of the type R_4S: where the lone pair of electrons was included as a ligand. There is a clear distinction between such "pentacoordinate" intermediates and the ones like **10** which are discussed in this review.

(13) (a) F. P. Boer, J. J. Flynn, E. T. Kaiser, O. R. Zaborsky, D. A. Tomalia, A. E. Young, and Y. C. Tong, *ibid.*, **90**, 2970 (1968); (b) F. P. Boer and J. J. Flynn, *ibid.*, **91**, 6604 (1969); (c) E. B. Fleischer, E. T. Kaiser, P. Langford, S. Hawkinson, A. Stone, and R. Dewar, *Chem. Commun.*, 197 (1967).

Reactions of Imidazoles with Cyclic Sulfate and Sulfonate Esters

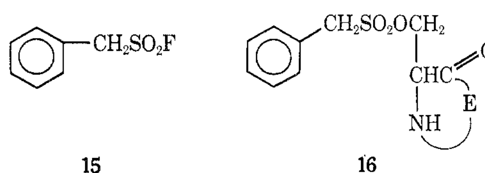
We have extended the range of catalysts whose action in the hydrolysis of cyclic sulfate and sulfonate esters we have investigated to include the bases imidazole and N-methylimidazole.[14] Catalysis by these bases was studied in water and deuterium oxide. The ratio of the second-order rate constants for attack by imidazole, $k_{Im}^{H_2O}/k_{Im}^{D_2O}$, in water and deuterium oxide, respectively, was found to be 3.6 for catechol cyclic sulfate (**1**) and 4.2 for 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone (**12**) at 25°. The ratio $k_{MeIm}^{H_2O}/k_{MeIm}^{D_2O}$ in the case of N-methylimidazole was 3.5 for the reaction of **1** and 3.5 for **12**.



In the case of carboxylic esters, which have been the most thoroughly studied systems, both the magnitude of the deuterium solvent isotope effect and the detection of an acylimidazole have been used to distinguish between nucleophilic and general base catalysis of hydrolysis.[15] From our observations of the solvent isotope effects in the hydrolytic reactions of **1** and **12** catalyzed by imidazole and N-methylimidazole, it appears that the imidazoles function here as general bases rather than as nucleophilic catalysts. We believe these to be the first examples of general base catalysis by organic catalysts in the hydrolyses of sulfate or sulfonate esters. As will be discussed below, we have found that the enzyme α-chymotrypsin catalyzes the hydrolysis of the sultone **12**.[16] This enzymatic reaction probably involves the participation of the imidazole ring of histidine-57 as a general base catalyst. Thus, there appears to be some similarity between the enzymatic and nonenzymatic catalytic action of imidazole in the hydrolysis of a sulfonate ester.[17]

Reactions of Enzymes with Cyclic Sulfate and Sulfonate Esters

In view of the remarkable lability of the aromatic five-membered cyclic sulfate and sulfonate esters to attack by hydroxide ion (and by other nucleophiles) we felt that it would be worthwhile to determine whether the esters might have an enhanced reactivity with the active sites of enzymes. This could possibly permit their use as reagents for the titration of enzyme active sites and for the elucidation of enzymatic reaction mechanisms. There was some foundation for these hopes since previous workers had found that sulfonylating agents such as α-toluenesulfonyl fluoride (**15**) react selectively in a stoichiometric fashion with the serine residue at the active site of α-chymotrypsin.[18] When **15** reacts with α- chymotrypsin a fully inhibited species, α-toluenesulfonyl-α-chymotrypsin (**16**), is formed, and the enzyme is stable in this modified inactive form over the pH range 3 to 8.5 under the usual conditions of assay.[18] When we treated α-chymotrypsin with 2-



hydroxy-α-toluenesulfonic acid sultone (**3**) the sulfonyl-enzyme **17** was produced. Unlike all previously reported sulfonylchymotrypsins,[18,19] 2-hydroxy-α-toluenesulfonyl-α-chymotrypsin (**17**) desulfonylates at neutral pH. Since **17** is structurally similar to the sulfonyl-enzyme **16** except for the presence of an *o*-hydroxyl group in **17**, our results suggest that the *o*-hydroxyl group of **17** must play a crucial role in the decomposition of this sulfonyl-enzyme.[20] An extensive kinetic analysis of the reaction of **3** with α-chymotrypsin was not attempted because the nitro-substituted sultone **12**, which is a much more convenient reagent for spectrophotometric measurements, was found to react rapidly with the active site of α-chymotrypsin to give the sulfonyl-enzyme **18** which is inactive toward both the active-site titrant cinnamoylimidazole[21] and the specific ester substrate N-acetyl-L-tryptophan methyl ester.

(14) K. W. Lo, K. Kudo, W. Berg, and E. T. Kaiser, manuscript in preparation.

(15) T. C. Bruice and S. Benkovic, "Bioorganic Mechanisms," Vol. 1, W. A. Benjamin, Inc., New York, N. Y., 1966, pp 46–66.

(16) J. H. Heidema and E. T. Kaiser, manuscript in preparation.

(17) It is interesting to ask why the imidazole- and N-methylimidazole-catalyzed hydrolyses of **12**, for instance, do not proceed by nucleophilic attack of the imidazole species on the sulfur atom to give intermediates like the sulfonylimidazole **13**, followed by hydrolysis of the intermediates regenerating the imidazoles and giving the sulfonic acid. The excellence of the leaving group formed when the sultone ring in **12** is broken might lead one to expect on the basis of our knowledge of the imidazole catalysis of carboxylic ester solvolyses that nucleophilic catalysis of the solvolysis of **12** by the imidazoles rather than general base catalysis should occur. It is important therefore to mention the possibility that nucleophilic attack by imidazole to give the sulfonylimidazole **13** may indeed occur, but we may not detect this attack. The reason why the formation of **13** may not be detected is that **13** may recyclize to form the starting sultone, **12**, much faster than it hydrolyzes to give the product sulfonic acid, **14**. In that event it would be entirely conceivable that imidazole and N-methylimidazole can attack 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone by both nucleophilic and general base catalyzed routes, but only the latter pathway contributes significantly to the observed hydrolysis of the cyclic sulfonate.

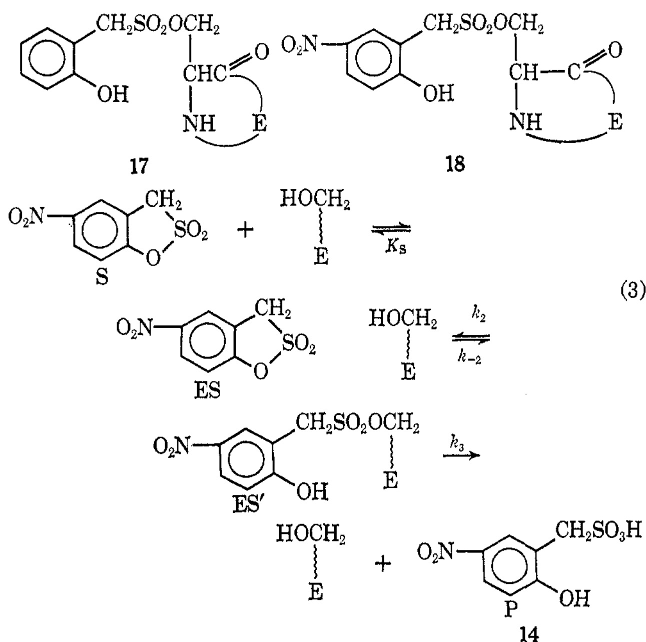
(18) D. E. Fahrney and A. M. Gold, *J. Amer. Chem. Soc.*, **85**, 997 (1963); A. M. Gold and D. Fahrney, *Biochemistry*, **3**, 783 (1964); A. M. Gold, *ibid.*, **4**, 897 (1965); A. M. Gold and D. Fahrney, *ibid.*, **5**, 2911 (1966).

(19) V. Massey, W. F. Harrington, and B. S. Hartley, *Discuss. Faraday Soc.*, **20**, 24 (1955); B. S. Hartley and V. Massey, *Biochim. Biophys. Acta*, **21**, 58 (1956); G. Gundlach, C. Kohne, and F. Turba, *Biochem. Z.*, **336**, 215 (1962); J. Kallos and D. Rizok, *J. Mol. Biol.*, **9**, 255 (1964); D. Rizok and J. Kallos, *Biochem. Biophys. Res. Commun.*, **18**, 478 (1965).

(20) (a) J. H. Heidema and E. T. Kaiser, *J. Amer. Chem. Soc.*, **89**, 460 (1967); (b) J. H. Heidema and E. T. Kaiser, *ibid.*, **90**, 1860 (1968); (c) J. H. Heidema, Ph.D. Thesis, Department of Chemistry, University of Chicago, 1969.

(21) G. R. Schonbaum, B. Zerner, and M. L. Bender, *J. Biol. Chem.*, **236**, 2930 (1961).

Kinetic studies of the sulfonylation and desulfonylation steps in the reaction of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone (**12**) with α-chymotrypsin have revealed that a reaction sequence related to that observed with carboxylic ester substrates is followed (eq 3).[20c,22]



The kinetics of the sulfonylation of α-chymotrypsin by the nitro-substituted sultone **12** showed a bell-shaped pH dependence for the function k_2/K_S (K_S is the dissociation constant for the Michaelis complex in eq 3) with pK_a values of 7.04 and 8.67 for the groups on the enzyme responsible for this behavior (Figure 1). Similar results have been obtained for a number of carboxylic ester substrates.[23] The group with a pK_a near 7.0 is presumably the imidazole ring of a histidine residue (histidine-57) which is catalytically active only in its unprotonated form. The ionizing function in α-chymotrypsin with a pK_a which generally appears to fall between 8.6 and 9.0 has been attributed in the recent literature to a group affecting binding, probably the α-amino group of an N-terminal isoleucine residue (isoleucine-16).[24,25]

The sulfonyl residue in **18** is ideally suited to be a "reporter molecule";[26] it is bound covalently (and reversibly, as shall be discussed shortly) to the very center of the active site. Absorbance data giving the

(22) M. L. Bender and F. J. Kezdy, *Ann. Rev. Biochem.*, **34**, 49 (1965).
(23) M. L. Bender, G. E. Clement, F. J. Kézdy, and H. d'A. Heck, *J. Amer. Chem. Soc.*, **86**, 3680 (1964).
(24) M. L. Bender, M. J. Gibian, and D. J. Whelan, *Proc. Nat. Acad. Sci. U. S.*, **56**, 833 (1966); M. L. Bender and F. C. Wedler, Jr. *J. Amer. Chem. Soc.*, **89**, 3052 (1967); P. Valenzuela and M. L. Bender, *Proc. Nat. Acad. Sci. U. S.*, **63**, 1214 (1969).
(25) H. L. Oppenheimer, B. Labouesse, and G. P. Hess, *J. Biol. Chem.*, **241**, 2720 (1966); A. Himoe, P. C. Parks, and G. P. Hess, *ibid.*, **242**, 919 (1967).
(26) M. B. Hille and D. E. Koshland, Jr., *J. Amer. Chem. Soc.*, **89**, 5945 (1967).

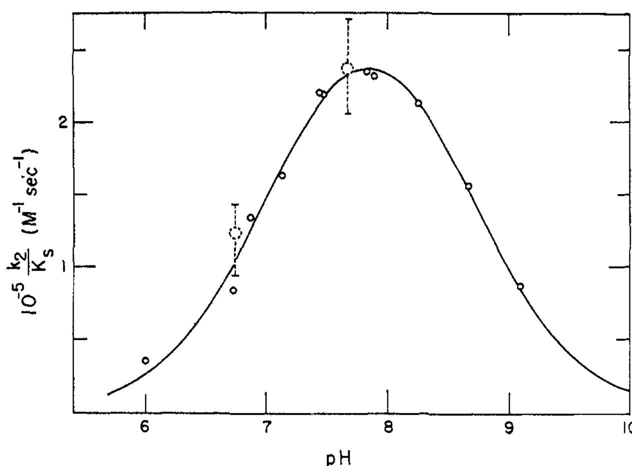
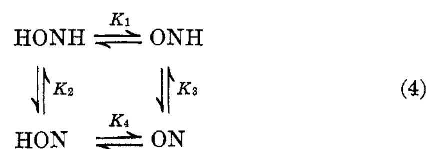


Figure 1. Rate-pH profile for the sulfonylation of α-chymotrypsin by 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone at 25.0°. The curve is a theoretical one for pK_1 = 7.04, pK_2 = 8.67, and k_2/K_S(lim) = 3.1 × 10^5 M^{-1} sec^{-1} (a computer-calculated, least-squares fit to the excess enzyme points; see ref 20b). The solid circles represent points obtained with enzyme in excess and the dotted ones are for points obtained with sultone in excess. The buffer solutions were of ionic strength 0.20 and contained 0.02% CH_3CN.

pH dependence for the ionization of the nitrophenol chromophore in **18** to produce the nitrophenolate species do not conform to the shape of a theoretical sigmoid curve.[20b] The ionization of the phenolic proton in the sulfonyl-enzyme is influenced by another ionization of a nearby group occurring at a similar pH.[27] The group perturbing the ionization of the phenol function is most likely the imidazole ring of histidine-57 of the active site. We have proposed the scheme of eq 4 to account for the ionization behavior of the nitrophenol chromophore in **18** where HONH is the sulfonyl-enzyme protonated at both the imidazole and the phenol groups, ON is the



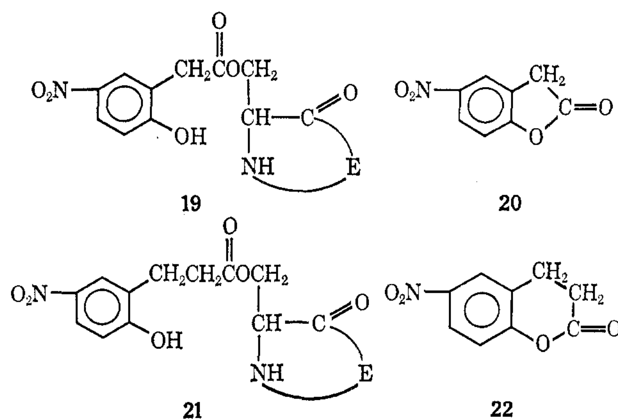
(4)

sulfonyl-enzyme with both groups deprotonated, and ONH and HON are singly protonated species at the imidazole and phenol, respectively. By the procedure outlined in ref 20b we have calculated the following pK values for the groups in eq 4: pK_1 = 6.75, pK_2 = 6.76, pK_3 = 7.57, and pK_4 = 7.56.[28]

We have observed one very important difference for the desulfonylation of **18** from the mechanistic pathway followed by the usual carboxylic ester substrates. The k_{-2} step of eq 3 leading to regeneration of the starting ester is significant in the case of the sulfonyl-enzyme **18** under conditions where the reversion of most acyl-enzymes to the corresponding starting esters is negligi-

(27) For a study of a related phenomenon in aminothiol compounds, see R. E. Benesch and R. Benesch, *ibid.*, **77**, 5877 (1955).
(28) These pK values have been revised somewhat from those given earlier in ref 20b.[20c] We should mention here also that we do not intend to imply that eq 4 uniquely accounts for the ionization behavior of the nitrophenol chromophore. However, it represents a reasonable postulate.

ble. In contrast to the general carboxylic ester substrates where the leaving alcohol moieties become part of the solvent when the acyl-enzymes are generated, the leaving alcohol in **18**, the phenolic group, remains in a sterically favorable position for attack on the sulfonyl function. Similar behavior has been observed in the case of the acyl-enzyme **19** formed from the reaction of the nitro-substituted lactone **20** with the active site of α-chymotrypsin.[29] On the other hand the acyl-enzyme **21** obtained from the nitro-substituted aromatic lactone **22** only deacylates by the usual k_3-type step and does not revert detectably to the starting ester by a k_{-2}-type step.[29] Thus, in the acylenzyme **21** the phenolic hydroxyl group does not seem to be in a favorable position for attack on the carbonyl function.



The rate of deacylation of the sulfonyl-enzyme **18** (*via* a combination of steps $k_{-2} + k_3$) was measured using a large excess of the specific substrate N-acetyl-L-tryptophan methyl ester which rapidly scavenges any free enzyme. The pH dependence of the first-order rate constants observed is illustrated in Figure 2. A computer-calculated[30] least-squares fit to the data gave $pM = 6.96$ and $pN = 7.64$, where M refers to the observed ionization constant on the acidic side of the profile and N refers to that on the alkaline side. Deacylation reactions of acyl-chymotrypsins usually exhibit a sigmoidal pH profile, one ionizing group of $pK_a \sim 7.0$ (presumably the imidazole ring of histidine-57) being implicated in the catalytic process. To account for the pH dependence of the kinetics of the desulfonylation of **18**, we have hypothesized that the reactive form of the sulfonyl-enzyme is species HON of eq 4. In other words the enzyme has the imidazole ring of histidine-57 unprotonated and the phenolic hydroxyl protonated in the state from which desulfonylation occurs. The constants M and N obtained from the data of Figure 2 are related to the ionization constants of eq 4 by eq 5 and 6, respectively.[20b] The agreement between the ionization constants calculated from the titration of the phenolic

$$M = K_1 + K_2 \qquad (5)$$

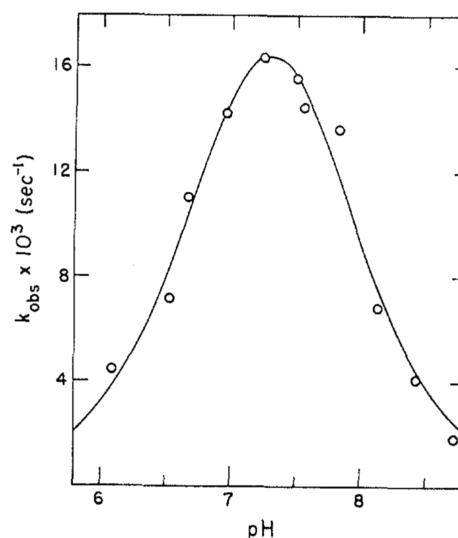


Figure 2. Rate-pH profile for the desulfonylation of 2-hydroxy-5-nitro-α-toluenesulfonyl-α-chymotrypsin (a combination of steps k_{-2} and k_3 of eq 3) at 25.0°. The curve is a theoretical one for $pK_M = 6.96$, $pK_N = 7.64$, and $k(\text{lim}) = 4.54 \times 10^{-2}$ sec^{-1} (a computer-calculated, least-squares fit). The buffers were of ionic strength 0.40 and contained 2.4% CH_3CN.

$$N = K_4/[1 + (K_1/K_2)] \qquad (6)$$

hydroxyl group in 2-hydroxy-5-nitro-α-toluenesulfonyl-α-chymotrypsin (**18**) and those found from the kinetics of desulfonylation is good.[31]

In the case of the acyl-enzyme **19** the principal pathway by which the carboxylic acid product is formed is by the regeneration of the starting lactone **20** which then undergoes a slow nonenzymatic hydrolysis.[29] A k_3-type step (see eq 3) does not appear to be very important for the acyl-enzyme **19**. However, we have found that regeneration of the starting sultone **12** followed by its nonenzymatic hydrolysis is not a sufficiently rapid process to account for the observed rate of formation of the product sulfonic acid **14**.[20c] Thus, the k_3 step of eq 3 cannot be neglected relative to k_{-2} in considering the desulfonylation of the sulfonyl enzyme **18**. The pH dependence of the k_3 step is similar to that shown in Figure 2.[20b,c,32]

The reactions of the cyclic sulfonate ester **12** with other enzymes have been and continue to be investigated in our laboratory. For example, this sultone appears to be the most rapidly hydrolyzed ester substrate of the zinc-containing metalloenzyme carbonic anhydrase known.[33] Studies with bovine carbonic anhydrase indicate that an ionizable group in the enzyme with a pK of 7.3 is involved in the carbonic anhydrase catalyzed hydrolysis of **12**. Similar observations have been reported previously for the pH–rate behavior of the carbonic anhydrase catalyzed hy-

(29) P. Tobias, J. H. Heidema, K. W. Lo, E. T. Kaiser, and F. J. Kézdy, *J. Amer. Chem. Soc.*, **91**, 202 (1969).

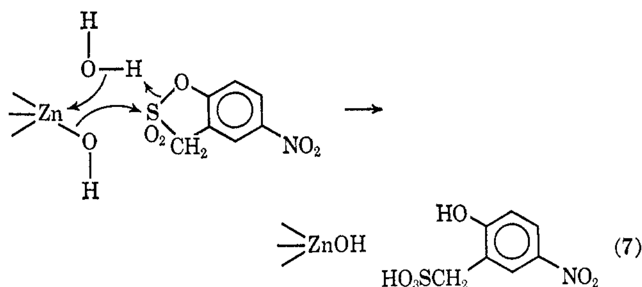
(30) A computer program written by Dr. P. L. Hall was used. See P. L. Hall, Ph.D. Thesis, University of Chicago, 1967.

(31) The titration and kinetic results agree well within the calculated standard deviations.[20b]

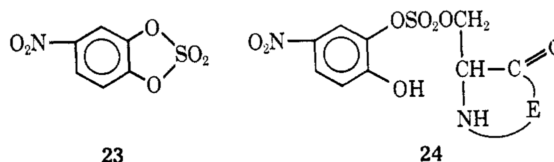
(32) The mechanistic role of the *o*-phenolic hydroxyl group of **18** in the k_3 step of eq 3 is unclear. One possibility is that the phenolic group might be acting as a general acid catalyst in the formation of the sulfonic acid product **14**.

(33) K. W. Lo and E. T. Kaiser, *Chem. Commun.*, 834 (1966); E. T. Kaiser and K. W. Lo, *J. Amer. Chem. Soc.*, **91**, 4912 (1969).

dration of CO_2,[34a] hydration of carbonyl compounds,[34b] and hydrolysis of nitrophenyl esters of carboxylic acids.[34c] The carbonic anhydrase catalyzed hydrolysis of **12** is subject to sulfonamide inhibition, as are the other reactions just mentioned. On the basis of our observations taken in conjunction with those of other investigators we have proposed the cyclic mechanism of eq 7 in which a zinc-bound hydroxide ion is the active catalytic species.[35]



The enzymatic reactions of other cyclic sulfate and sulfonate esters are also being studied. For instance, the cyclic sulfate **23** rapidly sulfonates the active site of α-chymotrypsin to produce **24**. Unlike the sulfonyl-enzyme **18**, however, the rate of decomposition of **24** is negligible over a wide pH range.[36]



Many additional aspects of the enzymatic hydrolyses of the reactive five-membered cyclic sulfates and sulfonates are being explored,[37] but to maintain the moderate length of this Account further discussion of our findings must be deferred. At this stage of our work, however, it certainly appears that these compounds and related cyclic esters will have wide applicability to the investigation of enzymatic reaction mechanisms.

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(34) (a) J. C. Kernohan, *Biochim. Biophys. Acta*, **81**, 346 (1964); (b) Y. Pocker and J. E. Meany, *Biochemistry*, **4**, 2535 (1965); (c) Y. Pocker and J. T. Stone, *ibid.*, **6**, 668 (1967).

(35) We favor the cyclic reaction pathway of eq 7 because it avoids the difficulty of postulating a net proton transfer from the enzyme to the solvent, a reaction which may be too slow to be consistent with the high catalytic efficiency of carbonic anhydrase. Although a step equivalent to that shown in eq 7 can be written for the carbonic anhydrase catalyzed attack of water on carbon dioxide, we do not mean to imply that additional steps might not be needed to account for the overall enzyme catalyzed equilibration of carbon dioxide and bicarbonate in solution.

(36) The reasons for this difference in the behavior of **18** and **24** are under examination.

(37) The use of the sultone **12** as an active-site titrant has been described: J. H. Heidema and E. T. Kaiser, *Chem. Commun.*, 300 (1968); F. J. Kézdy and E. T. Kaiser, "Principles of Active Site Titration of Proteolytic Enzymes," a review which will appear in a forthcoming volume of *Methods Enzymol.*

Biosynthesis of the Indole Alkaloids

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In the annals of biogenetic theory perhaps no single class of natural product has enjoyed more ingenious speculation from the organic chemist than the family of indole alkaloids, which are formally derived from the combination of tryptamine and an ubiquitous "C_9–C_{10}" unit. Not only the biochemical origin of the latter species but its appearance in the well-known *Corynanthe–Strychnos* pattern (**1**) have provoked stimulating comment ever since Barger[1] drew attention to a possible biogenesis of yohimbine in 1934.

Recent structural studies have increased the number of these alkaloids to more than 800, and two further main groups (with many subdivisions) can be discerned in which the C_9–C_{10} unit conforms to the *Aspidosperma* (**2**) and *Iboga* (**3**) skeletons.[2] Typical examples of these categories are ajmalicine (*Corynanthe*) (**4**), akuammicine (*Strychnos*) (**5**), vindoline (*Aspidosperma*) (**6**), and catharanthine (*Iboga*) (**7**), all of which occur (together with at least 70 other indole alkaloids) in the tropical periwinkle, *Vinca rosea.*[2] It is perhaps surprising that such prolonged biogenetic stimulation had, until 5 years ago, provided the experimental facts that although tryptophan served as a specific precursor for the appropriate (tryptamine) segment of the alka-

(1) G. Barger, IXth Congress Internacional de Quimica Pura y Applica, Madrid, Conferencias de Introduccion, 1934, p 177.

(2) (a) M. Hesse, "Indolalkaloide in Tabellen," Springer, Berlin, Vol. I, 1964, Vol. II, 1968; (b) *Alkaloids*, **11**, 1 (1968).