It is interesting to note that our ρ values are quite different from those reported by Gilliom and Ward.[4] These authors used *t*-butyl hypochlorite as a *t*-butoxy radical source in competitive reaction conditions and reported ρ (with σ) at 40° to be -0.75. Interestingly there is a close resemblance between this value and the ρ reported for photochlorination of substituted toluenes ($\rho = -0.76$ with σ and -0.66 with σ^+ at 40°).[2]

We have no positive evidence to account for these discrepancies so far, but it is highly likely that ρ values derived from present data are directly related to the hydrogen abstraction reaction of *t*-butoxy radical. Identical ρ values for photochlorination of toluenes by chlorine[2] and by *t*-butyl hypochlorite[4] might suggest the involvement of the same propagating radical in both reactions, but there is strong evidence against this view.[13]

The discrepancies may be attributed to the differences in the mechanism (spontaneous decomposition *vs.* chain reaction), in the method (indirect *vs.* direct), or in other unknown factors. Recently Wagner and Walling[14] have pointed out that a complicated reaction took place in a competitive chlorination of the toluene–cyclohexane mixture with *t*-butyl hypochlorite by reasons which are still obscure. Related work is in progress.

Acknowledgment. We wish to thank Professors M. Kumada and O. Simamura for encouragement throughout the work and helpful discussions.

(13) C. Walling and A. Padwa, *J. Org. Chem.*, 27, 2976 (1962).
(14) P. Wagner and C. Walling, *J. Am. Chem. Soc.*, 87, 5179 (1965).

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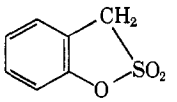
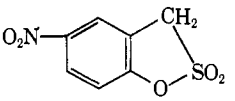
The Chymotrypsin-Catalyzed Hydrolysis of Sultones

Sir:

Recently we have been engaged in an investigation of the base-catalyzed hydrolyses of esters of sulfur-containing acids. We have found that certain five-membered cyclic sulfur-containing esters are extraordinarily labile to alkaline attack.[1,2] For example, the five-membered cyclic sulfonate, 2-hydroxy-α-toluenesulfonic acid sultone (I), undergoes hydroxide ion catalyzed hydrolysis 10^6 times faster than does the open-chain analog, phenyl α-toluenesulfonate. We now wish to report our observations on the unusual reactivity of the nitro-substituted sultone II[3] with a different type of nucleophilic species, the proteolytic enzyme α-chymotrypsin (CT), and the finding that II can be used to titrate the active sites of CT.



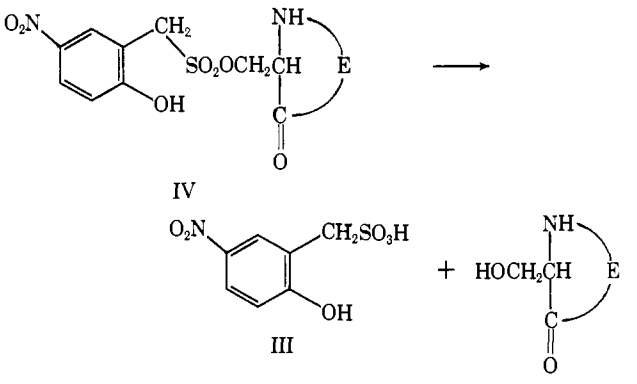
I



II

(1) E. T. Kaiser, I. R. Katz, and T. F. Wulfers, *J. Am. Chem. Soc.*, 87, 3781 (1965).
(2) O. R. Zaborsky and E. T. Kaiser, *ibid.*, 88, 3084 (1966).
(3) We thank Mr. K. W. Lo for a generous gift of II.

The hydrolysis of II to its product acid III (see below) was conveniently followed at pH values above 7 by spectrophotometrically monitoring the appearance of the phenolate ion peak near 400 mμ. CT does not absorb in this region. When II was added to a buffered solution containing CT (pH 7–8) a "burst" in the absorbance at 400 mμ, which was not observed in the absence of enzyme, occurred. The magnitude of this initial "burst" is determined by the species, enzyme or sultone, present in lower concentration. In the presence of excess enzyme, the "burst" was followed by a rise in absorbance to that of the product acid, III, at the given pH. These observations suggested that very rapid sulfonylation of the active site of CT by II to give the sulfonyl-enzyme IV occurred, followed by a slow decomposition of IV leading to the formation of the product III and regenerating the active enzyme.[4] We have performed several experiments which support this interpretation.



The absorbance "burst" has λ_{max} 390 mμ, whereas λ_{max} for the product acid is 410 mμ. At 410 mμ and pH 7.6 the maximum absorbance "burst" is four-sevenths of the product acid absorbance. The different λ_{max} values and extinction coefficients indicate that we are dealing with a distinct intermediate species. If the "burst" represents a stoichiometric 1:1 reaction giving rise to a sulfonyl-enzyme, IV, it could provide a titration method for the determination of the concentration of CT active sites similar to that presently available using N-*trans*-cinnamoylimidazole (CI).[5] Titration with II at higher pH values is complicated by relatively rapid spontaneous hydrolysis of the excess sultone, making extrapolation of the "burst" to zero time nonlinear. But, as with CI, a "burst" is also produced at pH 5, this time at 320 mμ, the phenol absorption peak. Titrations at pH 5.05 of enzyme stock solutions with CI and with II gave essentially identical values for the active site concentration.[6]

(4) In structure IV we have assumed that the site of attachment of the sulfonyl group to the enzyme is a serine hydroxyl group. Evidence presented by many other investigators suggests that this is true for acyl-, phosphoryl-, and sulfonyl-chymotrypsins. Some recent pertinent reviews are: M. L. Bender and F. J. Kézdy, *Ann. Rev. Biochem.*, 34, 49 (1965); T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," W. A. Benjamin, Inc., New York, N. Y., 1966, p 228–242.
(5) G. R. Schonbaum, B. Zerner, and M. L. Bender, *J. Biol. Chem.*, 236, 2930 (1961).
(6) The extinction coefficient of the intermediate may be found by adding a known amount of sultone II to an *excess* of the enzyme at the desired pH. All of the sultone is then consumed, resulting in a "burst" at 320 mμ (and/or 390 mμ depending on the pH), which corresponds to the formation of a known amount of sulfonyl-enzyme and when extrapolated to zero time allows the calculation of the extinction coefficient of this species.

Table I. The Desulfonylation of 2-Hydroxy-5-nitro-α-toluenesulfonyl-α-chymotrypsin (IV)[a]

E_0, M	S_0, M	k, sec^{-1}
7.77×10^{-5}	5.03×10^{-5}	6.78×10^{-4}
7.77×10^{-5}	5.03×10^{-5}	6.81×10^{-4}
7.77×10^{-5}	1.01×10^{-5}	6.63×10^{-4}
7.77×10^{-5}	1.01×10^{-5}	6.71×10^{-4}
1.55×10^{-5}	1.01×10^{-5}	6.77×10^{-4}

[a] At pH 7.58, 1.64% CH_3CN, and 25.0°.

We have examined the kinetics of the desulfonylation of IV at pH 7.58 and 25.0°. The results are indicated in Table I. Excess enzyme was used to eliminate the complications of spontaneous hydrolysis and turnover of the substrate. The kinetics were followed spectrophotometrically at 410 mμ, where $\Delta\epsilon$ is largest. All "runs" obeyed first-order kinetics, and the lack of any change in the rate constant, k, with fivefold variations in enzyme and sultone concentrations removes any doubts about the first-order nature of the observed reaction. Titration and dialysis experiments have also been performed, indicating: (1) that sultone-treated enzyme and native enzyme have identical activities after standing several hours at room temperature; (2) that CI and II attack the same active site,[7] and (3) that there is no detectably permanent incorporation of a chromophoric group. Preliminary results obtained with the unsubstituted sultone, I, show that it too reacts rapidly with chymotrypsin to form a sulfonyl-enzyme which then desulfonylates.[8]

Our observations that the sulfonyl-chymotrypsins produced by the reaction of CT with I and II desulfonylate at pH 7–8 and 25° are in remarkable contrast to the situation found when α-toluenesulfonyl fluoride reacts with the enzyme. In the latter case a fully inhibited species, α-toluenesulfonyl-chymotrypsin, is formed, and desulfonylation does not occur under conditions at which the enzyme is normally active.[9] An important question which arises is whether the phenolic hydroxyl group in IV must be in its protonated form in order for desulfonylation to occur. In an attempt to answer this question we have examined the pH dependence of the rate constant for desulfonylation. As indicated in Figure 1 the desulfonylation pH–rate profile is bell shaped, in contrast to the sigmoid profiles usually observed in the deacylation of acyl-CT,[10] and ionizing groups with pK's of 6.47 and 7.95 appear to be implicated in the desulfonylation reaction. The pK of 6.47 can be reasonably assigned to the ionization of a histidine residue.[11] However, the assignment of the

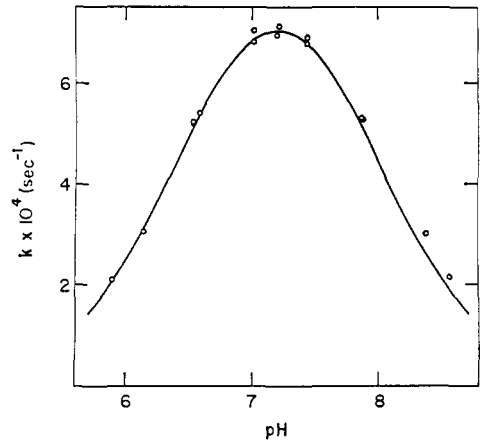


Figure 1.

pK of 7.95 is unfortunately very ambiguous since direct spectrophotometric measurements on the ionization constant of the phenolic hydroxyl in IV give a pK value of 7.2 for this group. One possible explanation for these observations is that the pK of 7.95 is attributable to the ionization of a group on the enzyme rather than to that of the substrate's hydroxyl. Another is that the pK of the phenolic hydroxyl in IV measured directly is different from the kinetically determined value because some change (*i.e.*, a conformational change) occurs in the desulfonylation step so that the pK of the hydroxyl group is perturbed during reaction.

We hope to elucidate the mechanism by which the sulfonylation and desulfonylation reactions of chymotrypsin take place.

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(7) In a typical experiment an excess of CI was added to a CT solution at pH 8. When II was added to this solution no "burst" whatsoever was observed at 390 mμ. The cinnamoyl-enzyme apparently will not form a sulfonyl-enzyme. In a complementary experiment a sixfold molar excess of the sultone I was added to a CT solution, and this solution was allowed to stand about 6 min to ensure the development of steady-state conditions. Although titrations are somewhat less accurate at this higher pH, addition of CI and II to separate samples of this solution showed that excess I cut the concentration of active sites accessible to reaction with *both* CI and II to the same extent. These experiments together with the titration results at pH 5 provide us with an excellent basis for concluding that CI and the sultones attack the same serine residue at the active site (see ref 4).

(8) Chymotrypsin does not appear to react with the acyclic compound *p*-nitrophenyl α-toluenesulfonate.

(9) A. M. Gold and D. Fahrney, *Biochemistry*, **3**, 783 (1964).

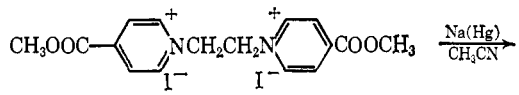
(10) M. L. Bender, G. E. Clement, F. J. Kézdy, and D. d'A. Heck, *J. Am. Chem. Soc.*, **86**, 3680 (1964).

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

A Pyridinyl Diradical. Preparation and Association

Sir:

We report here the preparation of the stable diradical 1,1′-ethylenebis(4-carbomethoxypyridinyl) (**1**) which exists as a triplet (epr) at 77°K. Radical–radical association produces dimers or polymers without covalent bonds, but with reduced paramagnetism. The ends of the *n*-mer behave like monoradicals,[1] as in the case of Chichibabin's hydrocarbon.[2]



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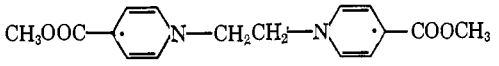

1

(1) E. M. Kosower and E. J. Poziomek, *J. Am. Chem. Soc.*, **86**, 5515 (1964).

(2) R. K. Waring, Jr., and G. J. Sloan, *J. Chem. Phys.*, **40**, 772 (1964).