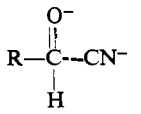
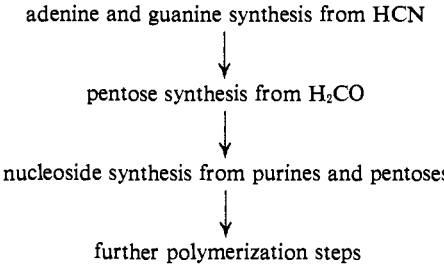
overall equilibrium in aqueous solution (K), in turn, is determined by the value of K_2, the acidity of the cyanohydrin hydroxyl (K_3) and the extent of hydration of the free aldehyde. The almost equal and opposite values of ρ^* for k_2 and k_{-2} are consistent with a transition state lying half-way along the reaction coordinate from reactants to product.



Prebiotic Significance. The great stability of glyconitrile and the expected stability for glycinenitrile show that glycolic acid and glycine could have been synthesized in the primitive oceans under very dilute conditions. Thus, if the free HCN concentration were, for example, 2×10^{-5} M, then 90% of the formaldehyde would have been in the form of glyconitrile. Conversely, if the free formaldehyde concentration were 2×10^{-5} M, then 90% of the cyanide would also have been in the form of glyconitrile. Once the nitrile has been hydrolyzed to the amide or the acid, the carbon–carbon bond is stable. Therefore, glycolic acid and glycine synthesis could have taken place at great dilution in the primitive oceans.

The proposed prebiotic synthesis of adenine requires high concentrations of hydrogen cyanide,[3] while the proposed prebiotic syntheses of sugars require high concentrations of formaldehyde.[4] The great stability of glyconitrile shows that the two type of compounds could not have been synthesized at the same time on the primitive earth unless there was a mechanism to concentrate the formaldehyde and hydrogen cyanide in different areas. It is more plausible to think that the adenine was synthesized during one period and the sugars during another period. Since sugars are decomposed rapidly on the geological time scale even at neutral pH and low temperatures, the likely sequence of reactions is



Acknowledgments. We thank Drs. Charles L. Perrin, Robert Fahey, and James E. Van Trump for helpful discussions. This work was supported by National Science Foundation Grant No. 25048.

Reaction of a Six-Membered Cyclic Sulfonate Ester, β-(2-Hydroxy-3,5-dinitrophenyl)ethanesulfonic Acid Sultone, with the Active Site of Papain[1]

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Contribution from the Department of Chemistry, University of Chicago, Chicago, Illinois 60637. *Received October 26, 1972*

Abstract: Rate constants for the sulfonylation of the thiol group at the active site of papain by the reactive aromatic six-membered sultone β-(2-hydroxy-3,5-dinitrophenyl)ethanesulfonic acid sultone (**2d**) to give an enzymatic thiolsulfonate species **5** have been measured over the range pH 3.5–9.6. The rate data obtained below pH 7 have been interpreted as indicating that, as with specific substrates, reaction occurs with Cys-25 in the thiol form assisted by general base catalysis by the basic form of a group in the enzyme with $pK_a \cong 4$. At higher pH values, nucleophilic displacement at the sulfonyl group of the sultone by the thiolate form of Cys-25 becomes predominant. The stoichiometric reaction of papain with **2d** to produce an enzyme-bound dinitrophenolate chromophore can be used for the titration of the active site of the enzyme. Reporter group titration of the dinitrophenolate chromophore present in the thiolsulfonate species **5** indicates that the ionization of the phenolic hydroxyl is significantly perturbed by that of a group of similar pK_a at the active site of papain. Most importantly, it has been found that at pH 5.2, recyclization to generate the sultone **2d** *via* the nucleophilic attack of the phenolic hydroxyl group in **5** on the sulfonyl function competes effectively with hydrolysis, the first observation of this kind for an enzyme other than a serine proteinase.

A variety of labile cyclic compounds, including lactones, sultones, cyclic sulfate and phosphate esters, react readily with the active sites of proteolytic enzymes. For example, the serine proteinase, α-chymotrypsin (α-CT), reacts with the family of compounds **1**–**2**.[2] In each case, as illustrated in eq 1 for the five-membered cyclic esters (where E·S represents the Michaelis complex), the group Y undergoes nucleophilic attack by the active site serine with formation of the acyl, sulfonyl, sulfuryl, or phosphoryl enzyme, as well as a new phenolic hydroxyl group.

The presence of this new group at the enzyme active site gives rise to two types of unusual reactions. First, regeneration of active enzyme may take place by a recyclization reaction (step k_{-2}) under conditions where kinetic control allows reformation of **1**, rather than **4** which is often thermodynamically more favored.

(1) A preliminary account of this work has appeared: P. Campbell and E. T. Kaiser, *Biochem. Biophys. Res. Commun.*, **4**, 866 (1972).

(2) (a) E. T. Kaiser, *Accounts Chem. Res.*, **3**, 145 (1970), and references therein; (b) E. T. Kaiser, T. W. S. Lee, and F. P. Boer, *J. Amer. Chem. Soc.*, **93**, 2351 (1971); (c) G. Tomalin, M. Trifunac, and E. T. Kaiser, *ibid.*, **91**, 722 (1969).

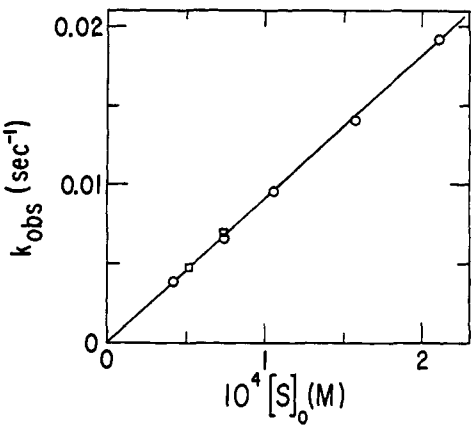
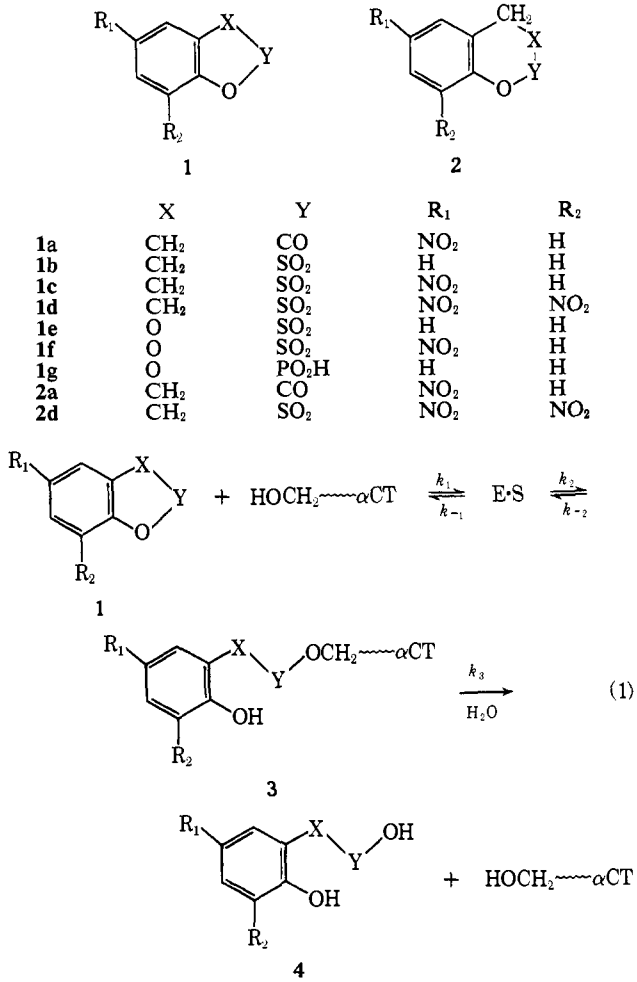


Figure 1. Pseudo-first-order rate constants for the sulfonylation of papain by sultone **2d** as a function of substrate concentration. The medium is 0.05 *M* acetate buffer containing 3.8% (v/v) CH_3CN, pH 5.20, μ = 0.15. (○) $[E]_0 = 3.60 \times 10^{-6}$ *M*; (□) $[E]_0 = 1.80 \times 10^{-6}$ *M*.



This type of reaction, observed with compounds **1a**,[2a] **1c**,[2a] and **1g**,[2b] parallels formation of the less stable virgin soybean trypsin inhibitor at the active site of trypsin, facilitated by intramolecular attack of an amino group present in the enzyme–inhibitor complex.[3] Second, the k_3 step is known to proceed in sulfonyl-chymotrypsins derived from **1b** and **1c**,[2a] while

(3) M. Laskowski, Jr., and R. W. Sealock, *Enzymes*, *3rd Ed.*, **3**, 376 (1971).

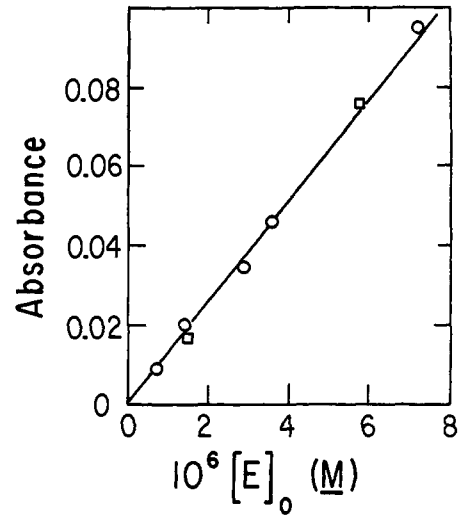


Figure 2. Equilibrium absorbance values at 400 nm produced by sulfonylation of papain by an excess of sultone **2d** are shown as a function of enzyme concentration. The medium is the same as for Figure 1: (○) $[S]_0 = 2.10 \times 10^{-4}$ *M*; (□) $[S]_0 = 1.05 \times 10^{-4}$ *M*.

analogous species with the phenolic hydroxyl group absent are totally inert.[4]

Recently, we have extended this study to another type of enzyme,[5] the sulfhydryl proteinase, papain.[6] This enzyme reacts with sultone **1c**, forming a thiolsulfonate intermediate which desulfonylates in a k_3 step directly analogous to the reaction of the sulfonyl-α-chymotrypsin formed from this sultone.[7] Again the desulfonylation is dependent on the presence of the new hydroxyl group, as other sulfonyl-papains are unreactive.[8] In this case, however, it is impossible to study a possible recyclization reaction because no accumulation of the thiolsulfonate intermediate is detectable, and because of a competing reaction, whereby the enzyme is irreversibly modified. In the present work, we have been able to avoid both these difficulties by the use of sultone **2d**, which has led to the fruitful study of both the sulfonylation and the desulfonylation reactions.

Results

When a solution of the six-membered sultone, substrate **2d**, was mixed with a buffered solution of papain, with substrate (S) in large molar excess over enzyme (E), a rapid increase in the absorbance at 400 nm was observed. This reaction followed strictly pseudo-first-order kinetics. At pH 5.2, the observed rate constant showed a simple linear dependence on the substrate concentration and was independent of enzyme concentration (Figure 1). The equilibrium absorbance, on the other hand, was independent of substrate concentration and directly proportional to enzyme concentration (Figure 2). Similar results were obtained at pH 7.0. In no case was any "turnover" reaction observed.

Rate constants for chromophore development were measured at a single substrate concentration over a

(4) 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.*, **5**, 2911 (1966).
(5) P. Campbell and E. T. Kaiser, *Bioorg. Chem.*, **1**, 432 (1971).
(6) A. N. Glazer and E. L. Smith, *Enzymes*, *3rd Ed.*, **3**, 501 (1971).
(7) J. H. Heidema and E. T. Kaiser, *J. Amer. Chem. Soc.*, **90**, 1860 (1968).
(8) J. R. Whitaker and J. Perez-Villaseñor, *Arch. Biochem. Biophys.*, **124**, 70 (1968).

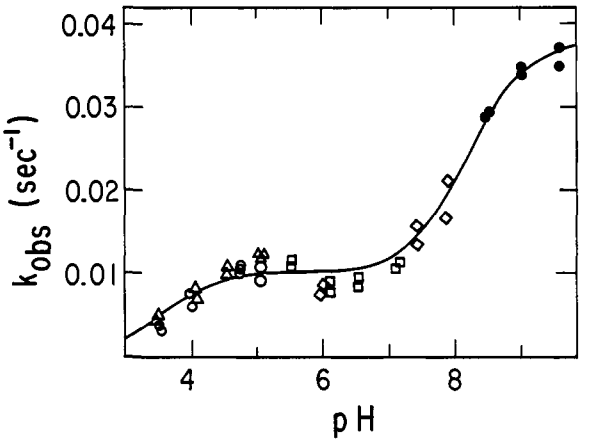


Figure 3. Pseudo-first-order rate constants for sulfonylation of papain by an excess of sultone **2d** as a function of pH. The buffers [(△) aspartate; (○) acetate; (□) MES; (◇) phosphate; (●) borate] all contained 3.8% (v/v) CH_3CN and had $[S]_0 = 1.26 \times 10^{-4}$ *M*. The solid line is calculated using the parameters of Table II.

large pH range. The pH–rate constant profile is shown in Figure 3. At pH values above 9.6 or below 3.4, irreproducible data were obtained because of irreversible denaturation of the enzyme.

The reaction was also carried out in the pH range 6–7 under conditions where $[E]_0 \cong [S]_0$. Figure 4 shows that the equilibrium absorbance no longer increases when $[E]_0 \geq [S]_0$. The data obtained under conditions where $[E]_0 > [S]_0$ are compiled in Table I, and indicate

Table I. Determination of the Extinction Coefficient of the Sulfonyl-Papain Species **5** at 400 nm

pH	Buffer	$10^5[E]_0$, *M*	$10^6[S]_0$, *M*	Absorbance	$10^4\epsilon$
7.0	Phosphate	3.12	6.87	0.0899	1.309
7.0	Phosphate	1.74	6.87	0.0884	1.287
7.0	Phosphate	0.75	6.87	0.0894	1.302
6.0	MES	2.35	6.87	0.0928	1.353
6.0	MES	1.17	6.87	0.0908	1.312
6.0	MES	1.17	6.87	0.0859	1.251
6.0	MES	2.35	4.80	0.0634	1.319
6.0	MES	2.35	3.43	0.0446	1.300
					Av 1.30 ± 0.03

that the extinction coefficient of the product is independent of pH (within this range), nature and concentration of buffer, and concentrations of starting materials.

Following the reaction of papain and excess **2d**, the products were separated by gel fitration. The protein fraction showed, relative to a similarly treated control, less than 1% activity toward the assay substrates ethyl *N*-benzoyl-L-argininate[9] (BAEE) and *p*-nitrophenyl *N*-benzoyloxycarbonylglycinate[10] (Z-glyPNP), and less than 2% sulfhydryl content.[11] In addition, the visible spectrum of the protein showed a new chromophore, λ_{max} 373, 405 nm. The ratio of the absorbance at 278 nm to that at 400 nm was 5.40 ± 0.06, and was the same for all protein fractions of the gel filtrate, indicating covalent attachment of a new group to the enzyme.

(9) J. R. Whitaker and M. L. Bender, *J. Amer. Chem. Soc.*, **87**, 2728 (1965).
(10) J. F. Kirsch and M. Igelström, *Biochemistry*, **5**, 783 (1966).
(11) G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).

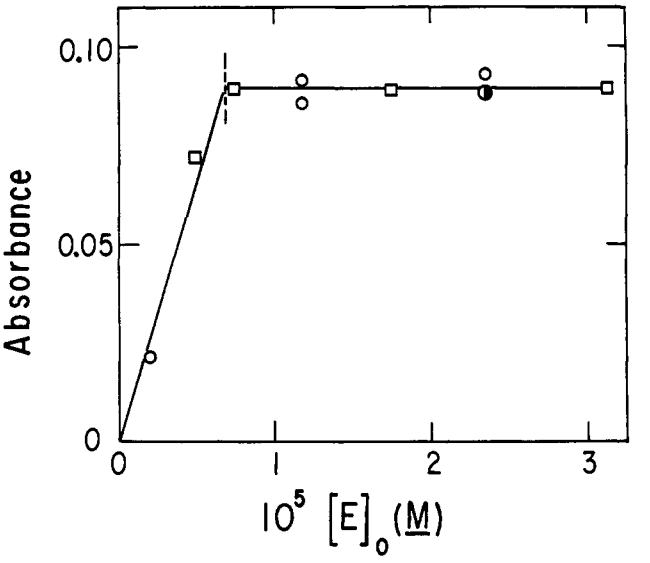


Figure 4. Determination of the extinction coefficient for the active site titration of papain by sultone **2d**. Equilibrium absorbances at 400 nm are shown as a function of $[E]_0$ in the region $[E]_0 \cong [S]_0$, with the dashed line marking $[E]_0 = [S]_0$. [E] is determined by rate assay calibrated against active site titration:[17] (○) 0.05 *M* MES buffer containing 3.8% (v/v) CH_3CN; (◑) same, except that $[S]_0$ was cut by half and the absorbance measurement was corrected; (□) 0.05 *M* phosphate buffer containing 3.8% (v/v) acetonitrile, $\mu = 0.15$.

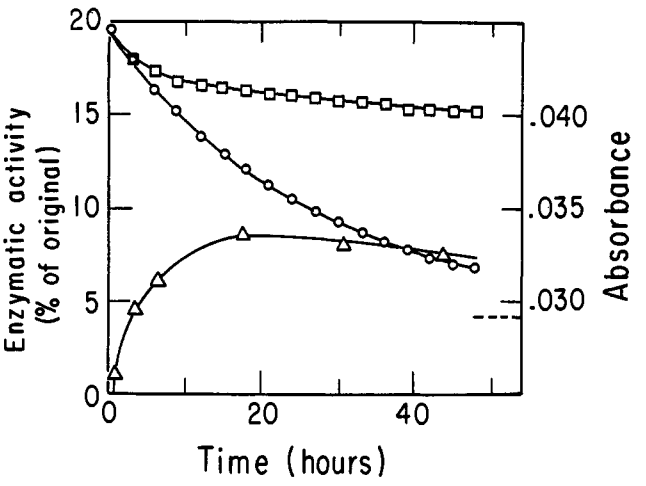


Figure 5. The reactions of **2d** in 0.05 *M* acetate buffer, pH 5.20, at 25°: (△) enzymatic activity as measured by standard assay; (□) absorbance change at 400 nm; (○) absorbance change when 0.028 *M* sodium chloroacetate is present; 0.003 *M* $HgCl_2$ produces an identical curve. The dashed line represents the calculated infinity absorbance of the chloroacetate reaction.

When the absorbance at 400 nm was measured as a function of pH, a maximum value was reached at pH 6 and above. The absorbance decreased at lower pH values down to 3, below which enzyme denaturation complicated measurements. It was, however, possible to determine that the pH dependence of the absorbance did not conform to a simple sigmoidal curve.

Some enzymatic activity (Z-glyPNP assay) was restored upon standing in pH 5.2 acetate buffer. A maximum activity of 8.6 ± 1.9% of the original activity was reached after 18 hr, at which time a slow and nearly linear decline in activity was observed (Figure 5).

The increase in enzymatic activity was paralleled

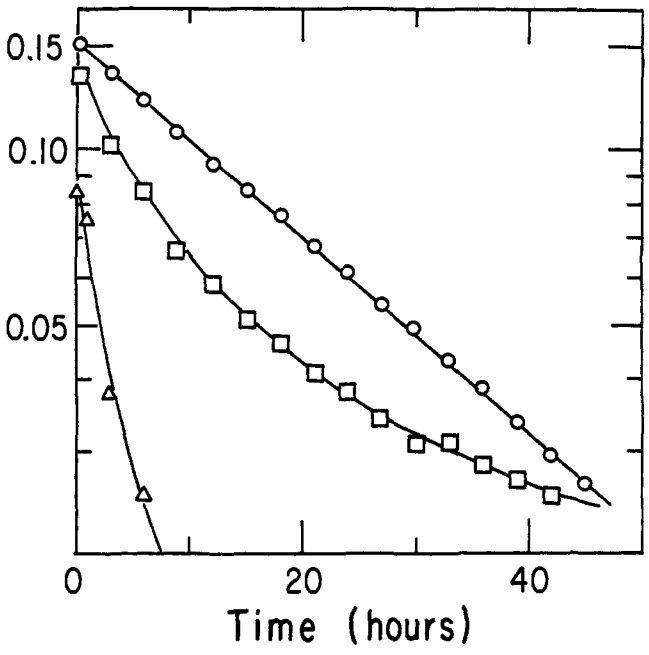


Figure 6. Semilogarithmic plot of the data of Figure 5. The ordinates are: (□) $30 \times (A - A_\infty)$ in the absence of inhibitors; (○) $10 \times (A - A_\infty)$ in the presence of chloroacetate; (△) activity$_{18\ hr}$ − activity (fraction of original).

by a decrease in the solution's absorbance at 400 nm. After 38 hr, the absorbance reached a constant value, which represented a loss of 11.3 ± 1.0% of the original chromophore. Ordinary semilogarithmic plots (Figure 6) show that both processes exhibit kinetic behavior which is between first and second order. Measurements of the first half-life are 4.5 ± 0.3 hr (absorbance) and 4 ± 1 hr (reactivation).

When mercuric chloride or sodium chloroacetate, both of which react rapidly and quantitatively with active papain,[6] were added to the sulfonyl–enzyme solution, different behavior was observed. Although the rates at which the enzyme was reactivated could not be studied, the absorbance change at 400 nm was cleanly first order (Figure 6) and of greater magnitude (Figure 5). Experimental points through the first 48 hr (*ca.* 3 half-lives) were used to calculate the absorbance at infinite time. This absorbance was 61.8 ± 1.2% of the initial value.

Under these conditions of enzyme scavenging, the reaction mixture was examined in several ways to characterize the products. A solution containing 5×10^{-6} *M* modified enzyme and 3×10^{-3} *M* $HgCl_2$ was allowed to stand in pH 5.2 acetate buffer for 4 days, then extracted with ether. Thin layer chromatography of the organic phase on silica gel plates showed only a single spot with two eluents. The R_f values, 0.2 in benzene, 0.45 in chloroform, were identical with those observed for an authentic sample of sultone **2d**. In contrast, a control extraction performed on a freshly prepared, gel-filtered solution of modified enzyme in like concentration showed no detectable ether soluble products.

Similar samples, treated with excess mercuric chloride or sodium chloroacetate for 3 days, were gel-filtered to isolate the protein. After correction for dilution, only 5 ± 2% of the chromophore absorbing at 373 and 405 nm remained bound to the enzyme. This is in quantitative agreement with the value (6%) predicted on the assumption that the cleavage reaction is governed by the same rate law as was observed for the absorbance decrease.

All enzymatic activity was abolished in the presence of inhibitors, and none was restored when they were removed by gel fitration after 3 days. But when these solutions were gently shaken with a large excess of *p*-toluenethiol in toluene and gel-filtered again, activity was restored to the extent indicated in Table IV.

Discussion

Nature of the Sulfonylation Reaction. The data of Figures 1 and 2 strongly suggest the following sequence.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot S \xrightarrow{k_2} EP \qquad (2)$$

Under conditions of substrate in large excess, the first-order rate constant for appearance of EP is given by eq 3, where $K_m = (k_{-1} + k_2)/k_1$. In the substrate

$$k_{obsd} = k_2(S)/(K_m + (S)) \qquad (3)$$

concentration range studied, a conventional double reciprocal plot, $1/k_{obsd}$ *vs.* 1/(S), passes, within experimental error, through the origin. Thus, we will be able to present only indirect evidence for binding of sultone **2d** to papain. We include the Michaelis complex, E·S, in the reaction scheme for completeness, and set a lower limit of 10^{-2} *M* on the value of K_m. Then to a very good approximation eq 4 holds, and if $k_{-1} > k_2$, this becomes eq 5 where $K_s = k_{-1}/k_1$.

$$k_{obsd} = (k_2/K_m)(S) \qquad (4)$$

$$k_{obsd} = (k_2/K_s)(S) \qquad (5)$$

pH Profile for Sulfonylation. For specific substrates of papain, reacting by the three-step sequence of eq 6,

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot S \xrightarrow{k_2} \underset{+}{ES'} \xrightarrow{k_3} E + P_2 \qquad (6)$$
$$\qquad\qquad\qquad\qquad\qquad\quad P_1$$

careful analysis[12] of the individual kinetic parameters indicates that K_s is independent of pH; k_2 shows a bell-shaped dependency, the rising limb with an inflection near pH 4.2–4.5, and the falling limb with one near 8.0–8.5; and k_3 follows a sigmoidal curve, rising at pH 4.2–4.5. While the identity of the group with pK_a = 4.2–4.5 is still an open question,[12,13] it is agreed that Cys-25 is required in its protonated form for enzymatic activity, accounting for the decrease in k_2 at pH 8.0–8.5.

The reaction of papain with irreversible alkylating agents such as the α-haloacetamides shows another type of pH dependence; k_2/K_s follows a sigmoidal curve, rising with a midpoint at pH 8.2–8.6.[14] This is interpreted as indicating a simple nucleophilic displacement with the thiolate form of Cys-25 as the reactive species.

In the case of the reaction of sultone **2d** with papain, the ordinate of Figure 3 shows a sigmoidal rise with a midpoint at a pH slightly below 4 and another with an inflection near pH 8.2. The simplest interpreta-

(12) E. L. Lucas and A. Williams, *Biochemistry*, **8**, 5125 (1969).
(13) M. L. Bender and L. J. Brubacher, *J. Amer. Chem. Soc.*, **88**, 5880 (1966).
(14) I. M. Chaiken and E. L. Smith, *J. Biol. Chem.*, **244**, 5087 (1969).

tion of these data is that below pH 7 the mechanism which applies to specific substrates is observed, with general base catalysis by the basic form of the group with $pK_a \cong 4$, and with the thiol in its protonated form. At higher pH values the nucleophilic reaction of the thiolate anion becomes predominant. This predicts the pH dependence of eq 7, where k_{gb} and k_n

$$\frac{k_{obsd}}{[S]} = \frac{k_{gb}/K_s}{1 + \frac{H^+}{K_1} + \frac{K_2}{H^+}} + \frac{k_n/K_s}{1 + \frac{H^+}{K_2}} \quad (7)$$

are the limiting values for collapse of the Michaelis complex by the general base catalyzed and nucleophilic pathways.

A computer calculated least-squares fit of the experimental points to this equation provides values of the parameters in Table II, implicating the same two ionizable groups as have generally been observed in papain reactions.

Table II. Kinetic Parameters for the Sulfonylation of Papain by Sultone **2d** at 25°

Parameter	Value (std dev), M^{-1} sec^{-1}
k_{gb}/K_s	80 (3)
k_n/K_s	301 (8)
pK_1	3.6 (0.2)
pK_2	8.21 (0.07)

Product of the Sulfonylation Reaction. The uv–visible spectrum of the gel-filtered product of the reaction between sultone **2d** and papain indicates the covalent attachment of an ionized dinitrophenol moiety to the enzyme. The data of Table III show that the stoichiometry of the reaction is simple, 1 mol of a dinitrophenoxide species being attached to 1 mol of papain. These data, plus the involvement of an ionizable group of $pK_a = 8.2$ in the reaction, the absence of a sulfhydryl group in the product, and the complete loss of enzymatic activity, show that sulfonylation of papain by sultone **2d** has taken place at the active site cysteine, with formation of a thiolsulfonate ester, according to eq 8.

Table III. Determination of the Stoichiometry of the Sulfonylation Reaction

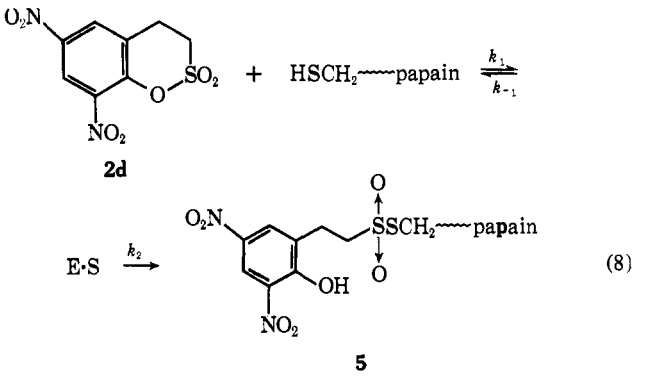
pH	10^6[protein]$_0$,[a] M	10^6[E]$_0$,[b] M	10^4[S]$_0$, M	A_{278}[c]	A_{400}[c]	Molar chromophore ratio[d]
5.20	5.32	4.42	2.52	1.201	0.210	1.07
5.20	5.32	4.42	1.26	1.192	0.208	0.95
5.20	4.44	3.69	1.26	0.981	0.171	0.97
6.91	5.32	4.42	2.52	1.159	0.217	1.02
6.91	5.32	4.42	1.26	1.208	0.226	1.04
6.91	4.44	3.69	1.26	1.003	0.188	0.91
						Av 0.99 ± 0.05

[a] Based on absorbance at 278 nm and ϵ 5.75 × 10^4 M^{-1} cm^{-1}. [b] By Z-glyPNP assay calibrated by active site titration with α-bromo-4-hydroxy-3-nitroacetophenone. [c] After dilution by gel filtration. [d] Dinitrophenoxide–papain, assuming only that a fraction of protein active toward the assay ester reacts with sultone, ϵ_{400} 13,000 M^{-1} cm^{-1} and $pK_a = 4$.

Model Studies of the Sulfonylation Reaction. In the absence of direct kinetic evidence for binding of substrate to the enzyme, the strongest evidence for the enzymatic nature of the sulfonylation reaction of papain comes from a comparison of its reaction rates to those of model sulfhydryl compounds. When glutathione, *N*-acetylcysteine, or 2-mercaptoethanol replaced papain, no detectable reaction could be observed at pH values below 8.[15] Under our conditions (**2d** = 7 × 10^{-5} M, RSH = 1 × 10^{-3} M) we would have observed a reaction with a second-order rate constant of 2 × 10^{-3} M^{-1} sec^{-1}, while the equivalent quantity for the reaction with papain, k_2/K_s, is about 10^2 M^{-1} sec^{-1} in this pH range. The most reasonable explanation for a rate acceleration of at least 50,000 is that enzymatic binding of the substrate takes place with probable general base catalysis of the attack by the thiol function of Cys-25 by the ionizable group with $pK_a \cong 4$.

Active Site Titration. The data of Figure 4 demonstrate that sultone **2d** reacts stoichiometrically with the active site of papain. Points on the flat part of the curve provide a value for the extinction coefficient of **5** of (1.30 ± 0.03) × 10^4 M^{-1} cm^{-1} at 400 nm. The amount of **5** formed when papain is treated with **2d** shows an excellent correlation with the enzyme's activity toward the specific substrate ethyl *N*-benzoyl-L-argininate[9] and with two other active site titrations for papain, using *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate[16] and α-bromo-4-hydroxy-3-nitroacetophenone,[17] respectively. The reaction of papain with **2d** thus qualifies as an active site titration itself.[18] Compared with the former titration procedure, it has the advantage of great simplicity and convenience, while it provides an optical change of over twice that of the latter at a wavelength where protein absorbance is vanishingly small. Still another advantage is the test for homogeneity of the enzyme furnished by the fit of the kinetic data for sulfonylation to the pseudo-first-order rate law.

Reporter Group Titration. The pH dependence of the absorbance of **5** at 400 nm does not follow a simple sigmoidal relationship, indicating that the absorbing chromophore must be perturbed by another group or groups at the active site. A simple model,[7] illustrated in eq 9 where OH represents the phenolic hydroxyl group in **5**, assumes one perturbing ionizable group,



(8)

(15) At pH 8 and above a side reaction occurs, which produces no dinitrophenoxide species. We have not investigated this reaction, but one possibility is aromatic nucleophilic substitution of the oxygen, as has been observed in a similar compound: W. Tagaki, T. Kurusu, and S. Oae, *Bull. Chem. Soc. Jap.*, **42**, 2894 (1969).

(16) M. L. Bender, M. L. Begué-Canton, R. L. Blakely, L. J. Brubacher, J. Feder, C. R. Gunter, F. J. Kézdy, J. V. Killheffer, Jr., T. H. Marshall, C. G. Miller, R. W. Roeske, and J. K. Stoops, *J. Amer. Chem. Soc.*, **88**, 5890 (1966).

(17) R. W. Furlanetto and E. T. Kaiser, *ibid.*, **92**, 6890 (1970).

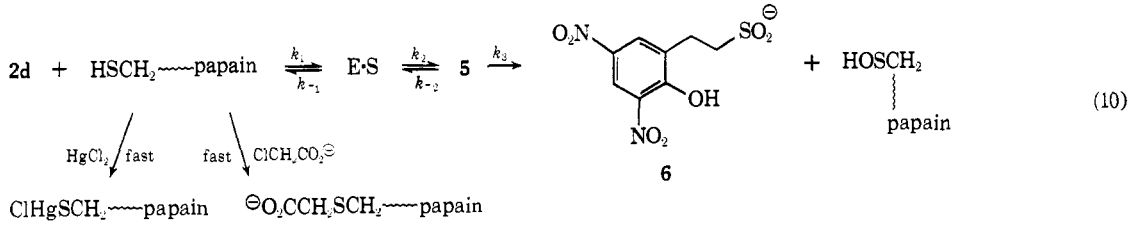
(18) F. J. Kézdy and E. T. Kaiser, *Methods Enzymol.*, **19**, 3 (1970).

called NH, and equal extinction coefficients for ONH and ON. A computer calculated least-squares fit of the experimental points for the absorbance of **5** in the pH range 3–6 in solutions of ionic strength 0.15 yielded values (standard deviations) for pK_1–pK_4 of 2.5 (0.3), 2.9 (0.3), 4.30 (0.05), and 3.93 (0.05), respectively.

$$\begin{array}{ccc} \mathrm{HONH} & \overset{K_1}{\rightleftharpoons} & \mathrm{ONH} \\ \downarrow\uparrow K_2 & & \downarrow\uparrow K_3 \\ \mathrm{HON} & \underset{K_4}{\rightleftharpoons} & \mathrm{ON} \end{array} \qquad (9)$$

The values of pK_1 and pK_2 are quite low. If the functional group on the enzyme with which the reporter group interacts is the kinetically important one, which normally exhibits a pK_a of about 4, and the model of eq 9 is correct, then our data seem to indicate that the phenolic species causes an appreciable increase in the acidity of that function. Since low values have also been obtained from the corresponding pK measurements in the reporter group titration of the sulfuryl-chymotrypsin produced from 4-nitrocatechol cyclic sulfate,[2c] any quantitative estimates of active site pK_a's based on this method must now be regarded with caution. In any event, it is clear that the dinitrophenoxide species present in **5** is interacting with at least one other group of similar pK_a at the active site of papain. Because the identity of the kinetically important group of $pK_a \cong 4$ at the active site of papain is uncertain and because it may be the group with which the reporter species is interacting, we are continuing to pursue our study of this interaction.

Desulfonylation Reactions. All the results obtained for the reactions of **5** are readily accounted for by the scheme shown in eq 10. The decomposition of **5**



(10)

occurs by two pathways, the k_{-2} and k_3 steps. Step k_{-2} represents nucleophilic attack on the thiolsulfonate by the phenolic hydroxyl in a fashion analogous to the attack on the serine sulfonate ester produced by the reaction of **1c** and α-chymotrypsin.[2a] Strong evidence for this step includes the actual isolation of **2d** in the reaction mixture from the decomposition of **5**, as well as the parallel decrease of the dinitrophenolate absorbance and increase in enzymatic activity in the absence of inhibitors. It is clear that the equilibrium constant $k_1k_2/k_{-1}k_{-2}$ is near unity under these conditions. In such a case the kinetics for approach to equilibrium would be complex,[19] and further complicated by the instability of free active enzyme over the time period of the experiment. The addition of enzyme scavengers effectively eliminates the k_1 reaction and thus both favors sultone formation and simplifies the kinetics.

(19) For $\mathrm{A} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} \mathrm{B} + \mathrm{C}$, $dB/dt = k_a(B_{eq} - B) + k_{-a}(B^2_{eq} - B^2)$ if $B = C$ throughout.

Table IV. Restoration of Enzymatic Activity[a] by Thiol Treatment[b]

Scavenger	Concn, M	Activity observed (%)	Activity predicted (%)
$HgCl_2$	0.006	91	100
$HgCl_2$	0.003	106	100
$ClCH_2CO_2^-$	0.028	55	62[c]
$ClCH_2CO_2^-$	0.014	48	60[c]

[a] Relative to activity before sulfonylation, based on Z-glyPNP assay. [b] 1000-fold excess of *p*-toluenethiol in toluene solution. [c] Assuming the entire absorbance decreased in the presence of chloroacetate represents irreversibly alkylated enzyme.

Postulation of step k_3 is necessary to explain the fact that not all of dinitrophenolate absorbance disappears during the desulfonylation of **5** even when scavengers are present. We have written the products as those which would result from the first step of normal thiolsulfonate hydrolysis.[5] While these products have not been identified, their formation is reasonable and in accord with our data. Sulfinate **6** would be expected to have visible spectral properties very similar to the corresponding sulfonate or sulfonyl enzyme. The existence of the enzyme sulfenic acid is more speculative as only a few such compounds have been isolated.[20] Evidence has recently accumulated, however, that sulfenic acids may be stable at the active sites of sulfhydryl enzymes.[21]

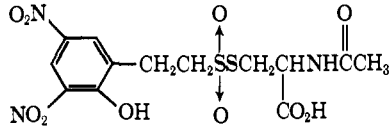
The scheme in eq 10 also quantitatively predicts the reactivation behavior upon treatment of the products with excess thiol. Enzyme inactivated by chloroacetate is unaffected, but that trapped by $HgCl_2$ or converted to the sulfenic acid form is returned to the active thiol form.[22] (If the sulfenic acid is itself unstable, likely products of its reaction would be eventually reduced to thiols.[23]) The agreement of expected and observed activities is shown in Table IV.

The rate constants of eq 10 have been estimated for the conditions given for Figure 5. The sum of k_{-2} and k_3 is simply the observed first-order rate constant for the absorbance change in the presence of enzyme inhibitor. Their ratio is calculated from the final absorbance, under the assumption that the k_3 step produces no absorbance change. This treatment yields values of $(4.0 \pm 0.5) \times 10^{-6}$ sec^{-1} and $(6.2 \pm 0.5) \times 10^{-6}$ sec^{-1} for k_{-2} and k_3, respectively. Measurement of the rate constants for the sulfonylation

(20) J. L. Kice and J. P. Cleveland, *J. Amer. Chem. Soc.*, **95**, 104 (1973), and references therein.
(21) A. N. Glazer, *Annu. Rev. Biochem.*, **39**, 101 (1970).
(22) Reactivation of mercuripapain by thiols is well known.[6] Sulfenic acids react with thiols to produce disulfides, which may then be further reduced; see D. Trundle and L. W. Cunningham, *Biochemistry*, **8**, 1919 (1969); D. J. Parker and W. S. Allison, *J. Biol. Chem.*, **244**, 180 (1969).
(23) J. L. Kice and G. B. Large, *J. Org. Chem.*, **33**, 1940 (1968).

reaction under the same conditions gives $k_2/K_s = 91 \pm 4\ M^{-1}\ sec^{-1}$, with $K_s \geq 10^{-2}\ M$.

The enzymatic nature of the reactions of the papain derivative **5** is demonstrated by the fact that the model thiolsulfonate **7** is completely stable for several days



7

at 25° in the pH range 2–10, in the presence and absence of the enzyme inhibitors used in this work. The recyclization reaction, furthermore, is different in kind from any known reaction of thiolsulfonates, which normally undergo nucleophilic attack only at the sulfenyl sulfur.[24] Moreover, the observation of recyclization with an enzyme other than a serine proteinase indicates the reasonableness of the hypothesis that such processes may be important in the reactions of naturally occurring cyclic esters and related compounds. Since recyclization requires the enzyme active site, and is not simply a result of favorable steric proximity of the hydroxyl group,[25] the previously postulated general acid catalysis,[2a,5] although not directly demonstrated here, appears even more likely.

Experimental Section

All spectrophotometric measurements were made on a Cary 15 or a Gilford 222 spectrophotometer. pH measurements were made on a Beckman 1226-A Research pH meter equipped with an Arthur H. Thomas 4858-L15 combination glass-calomel electrode. Calibrations were made against Fisher standard buffers. Thin layer chromatography was performed with Eastman Chromagram 6060 silica gel sheets.

Materials. Papain, Lot No. PAPIFA, was purchased from Worthington Biochemical Corp. Its handling has been described previously.[5]

β-(2-Hydroxy-3,5-dinitrophenyl)ethanesulfonic Acid Sultone (2d). A mixture of 1.2 ml of 70% nitric acid (19 mmol) and 2.0 ml of concentrated sulfuric acid was added dropwise to 310 mg (1.68 mmol) of β-(2-hydroxyphenyl)ethanesulfonic acid sultone[26] in 3.0 ml of concentrated sulfuric acid. Crushed ice was added slowly, forming a white precipitate which was filtered, washed with cold water, dried, and recrystallized from ethanol: yield 300 mg (64%); mp 186–187.5°; nmr (d_6-DMSO) δ 3.68 (m, 2), 4.16 (m, 2), 8.72 (m, 2); ir (KBr) 3125, 1180, 1095 cm^{-1}; uv (CH_3CN) 243 nm (ε 12,600 $M^{-1}\ cm^{-1}$).

Anal. Calcd for $C_8H_6N_2O_7S$: C, 35.04; H, 2.72. Found: C, 35,14; H, 2.31.

S-(β-(2-Hydroxy-3,5-dinitrophenyl)ethanesulfonyl)-N-acetyl-L-cysteine (7). Disodium Salt. **2d** (100 mg, 0.365 mmol) and 59.5 mg (0.365 mmol) of *N*-acetyl-L-cysteine were dissolved in 100 ml of acetonitrile. Sodium carbonate (38.6 mg, 0.365 mmol) was added and the mixture stirred at reflux for 18 hr. Solvent was removed and the residue recrystallized from ethanol to give 98 mg (56%) of product: mp 210° dec; nmr (D_2O) δ 2.02 (s, 3), 3.1 (m, 6), 5.6 (m, 1), 7.78 (d, J = 3 Hz, 1), 8.45 (d, J = 3 Hz, 1); ir (KBr) 3160, 1620, 1545, 1210, 1065 cm^{-1}; uv (H_2O) 369 nm (ε 8510 $M^{-1}\ cm^{-1}$).

Anal. Calcd for $C_{13}H_{13}N_3O_{10}S_2Na_2$: C, 32.44; H, 2.72; N, 8.73. Found: C, 32.52; H, 2.85; N, 8.80.

α-Bromo-4-hydroxy-3-nitroacetophenone[27] was prepared by bromination of the parent compound. Acetonitrile, J. T. Baker reagent grade, was distilled from phosphorus pentoxide. Water was distilled and then passed through a mixed-bed deionizing column. All other materials were commercially available and used without further purification.

Gel filtrations were routinely performed on a 15 × 1.6 cm column of Sephadex G25, purchased from Pharmacia. The null volume was determined and checked from time to time with Pharmacia Blue Dextran 2000. Calculations based on absorbance measurements indicated 93–100% recovery of protein samples. It was found that reproducibility required the use of two columns, one of which was reserved for solutions containing no thiols.

Buffers were prepared so that the buffering species always totaled 0.05 *M*; μ was held constant at 0.15 with sodium chloride. Exceptions were the zwitterionic buffers aspartic acid and 2-(*N*-morpholino)ethanesulfonic acid, to which no additional salt was added.

Kinetic Measurements. All reaction mixtures were thermostated at 25.0 ± 0.1°. The sulfonylation reaction was initiated by introducing, on a flat-tipped stirring rod, a measured volume of an acetonitrile solution of **2d** into a buffered enzyme solution in an open quartz cuvette. At higher pH values, where nonenzymatic hydrolysis of **2d** interfered, only the double-beam spectrophotometer was used, and a control reaction, with no enzyme present, was initiated in the reference compartment immediately before the enzymatic reaction was begun. The desulfonylation reactions were slow relative to the times required for their initiation.

Rate constants were computed by noting the absorbance values of at least 10 half-lives, and plotting $|A_\infty - A|$ *vs.* time on semilog paper. For the slow desulfonylation reactions, the infinity absorbance value was taken as the intersection of a plot of A_t *vs.* $A_{t+\Delta}$ with the line $A_t = A_{t+\Delta}$.[28] For the sulfonylation reactions at high pH, spontaneous hydrolysis of the sultone sometimes precluded a stable infinity reading, even when a reference reaction was run. In such cases, the absorbance change at long time was linear. The difference between the absorbance at any time and the extrapolation of the linear portion to that time was then plotted *vs.* time on semilog paper, and excellent straight lines were obtained.

Acknowledgment. The support of this research by a National Institutes of Health Postdoctoral Fellowship (P. C.), an Alfred P. Sloan Foundation Fellowship (E. T. K.), and a National Institutes of General Medical Sciences grant is gratefully acknowledged.

(24) A. J. Parker and N. Kharasch, *Chem. Rev.*, **59**, 583 (1959); S. Oae, R. Nomura, Y. Yoshikawa, and W. Tagaki, *Bull. Chem. Soc. Jap.*, **42**, 2903 (1969).

(25) Model studies with the oxygen analogs of **7** show simple SN2 displacement of the primary sulfonate as the only reaction: W. Berg, unpublished results.

(26) W. E. Truce and F. D. Hoerger, *J. Amer. Chem. Soc.*, **76**, 5357 (1954).

(27) G. Sipos and R. Szabo, *Acta Univ. Szeged., Acta Phys. Chem.*, **1**, 126 (1961).

(28) F. J. Kézdy, J. Jaz, and A. Bruylants, *Bull. Soc. Chim. Belg.*, 67, 687 (1958).