$SO_2Ph$ by a wide variety of nucleophiles in 60% dioxane. The $pK_a$ of the leaving group $PhSO_2^-$ is about 1.5, the sulfur is highly polarized, and the system is very reactive. The rate constants for attack at sulfonyl sulfur by 20 nucleophiles closely paralleled those for attack at carbonyl carbon of acetate esters with very good leaving groups. In neither Kaiser's work on sultones nor Kice's work could the question of direct displacement on sulfur *vs.* pentacovalent intermediates be resolved. For the $\alpha$-disulfones, either attack is rate limiting or there is no intermediate. Kice found an $\alpha$ effect of 72 for $HO_2^-/HO^-$. Our data show that hydroperoxide ion acts toward primary alkyl sulfonates like the vast bulk of nucleophiles, attacking entirely at carbon. However, the possibility of sulfonyl sulfur attack with some very hard nucleophiles or with favorable geometric alignments is by no means excluded. From the neopentyl methanesulfonate result, however, it is clear that the leaving group must be rather good for reaction to be of reasonable rate.

The enhanced nucleophilicity of $HO_2^-$ (*vs.* other oxygen nucleophiles) toward $sp^2$ (carbonyl and olefinic) carbon is well known and along with other examples of the $\alpha$ effect has been discussed recently.[17,18] At the outset of our research project, there was only one unambiguous example of enhanced nucleophilicity by an $\alpha$-effect nucleophile toward $sp^3$ carbon, that of $HO_2^-$ toward benzyl bromide, in which Pearson reported a ratio for $k_{HO_2^-}/k_{HO^-}$ of 35.[6] Several recent reports with other nucleophiles have shown that amine nucleophiles tend not to give enhanced reactivity toward $sp^3$ carbon but that oxygen nucleophiles do.[18,19] Our study confirms these observations and provides another value in agreement with those previously observed.

The lack of reactivity of the neopentyl system puts an upper limit upon the reactivity of sulfonate sulfur toward hydroperoxide ion for normal sulfonates ($k_2 < 5 \times 10^{-7}\ M^{-1}\ sec^{-1}$). The cyclic sulfonates are thus attacked at sulfur by $HO^-$ at $10^8$ times this rate and the $\alpha$-disulfones attacked by $HOO^-$ about $10^{10}$ times as fast.

(18) J. E. Dixon and T. C. Bruice, *J. Amer. Chem. Soc.*, **94**, 2052 (1972).
(19) J. E. Dixon and T. C. Bruice, *ibid.*, **93**, 6592 (1971); W. Richardson and V. Hodge, *ibid.*, **93**, 3996 (1971).

# Reaction of Phenylmethanesulfonyl-$\alpha$-chymotrypsin with Hydrogen Peroxide and Other Nucleophiles[1,2]

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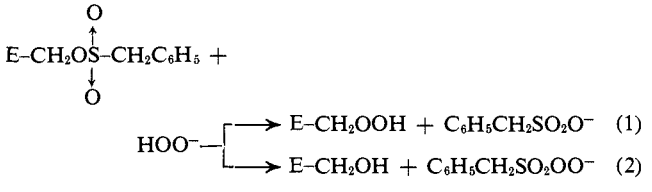
**Abstract:** Phenylmethanesulfonyl-chymotrypsin (PMS-CT), a stable catalytically inactive sulfonate ester of the active site serine of the enzyme, is desulfonated and reactivated by hydrogen peroxide. The reactivated material was isolated and subjected to a variety of analyses. Previously reported data indicating the presence of a hydroperoxy group at the site have now been compared to data from the same experiments using chymotrypsin incubated with hydrogen peroxide as a control, and there is now clear evidence that the reactivated material is normal hydroxy enzyme oxidized to the sulfoxide at methionine-192. The catalytic behavior and its pH dependence, the reactions with active site serine and histidine reagents, and amino acid analyses of reactivated and hydrogen peroxide oxidized chymotrypsin are identical. Preincubation of $\alpha$-chymotrypsin with hydrogen peroxide (generation of methionine-192 sulfoxide) neither retards sulfonation nor alters the stability of the sulfonyl enzyme in buffer alone. The rate of reactivation with peroxide is the same for preoxidized and native sulfonyl enzymes. Thus, hydrogen peroxide is reacting with the sulfonate group directly. The reactivation reaction itself shows dependence on a basic group of $pK_a \sim 7$, is overall first order (at high peroxide concentrations) in PMS-CT, and gives one $^{14}C$ loss for each active site appearing for [7-$^{14}$C]PMS-CT. A range of other nucleophiles including hydrazine, hydroxylamine, *N*-phenylbenzohydroxamic acid, phenol, and pyridine-2-aldoxime methiodide gave no loss of sulfonate. Hydrogen peroxide did not reactivate diisopropylphosphoryl-chymotrypsin. Methyl hydroperoxide, however, very slowly but quantitatively reactivates PMS-CT leading to one active site per sulfonate lost (by isotope labeling). This is shown to not be due to contamination by, or formation of, hydrogen peroxide. As shown by an isotope dilution and recovery experiment, phenyl methanesulfonate is the exclusive (and quantitative) other product in the hydrogen peroxide reaction. No persulfonate could be detected. These data, along with rates of reaction in comparison with model systems, show that the reactivation reaction involves direct attack on the sulfonate ester group to give ultimately serine enzyme and sulfonate ion, that the reaction is specific for hydrogen peroxide, and that the reaction is enzyme catalyzed. Possible pathways are discussed.

An earlier communication from this laboratory[3] described the reaction of catalytically inactive serine-195 sulfonated chymotrypsin (phenylmethanesulfonyl-chymotrypsin, PMS-CT) with hydrogen peroxide to produce a reactivated enzyme (react-CT). On the basis of organic primary sulfonate chemistry along with various aspects of the nature of the reactivation reaction, the loss of $^{14}C$ label from radioactive PMS-CT,

(1) (a) This research was supported by the Research Corporation and the Intramural Fund of the University of California. (b) Abstracted in part from the Ph.D. Theses of N. A. R., March 1973, and D. L. E., December 1972.
(2) Presented in part at the 162nd National Meeting of the American Chemical Society, Washington, D. C., Sept 1971, Abstract Biol-51. The current conclusions are described in this abstract.

(3) M. J. Gibian, D. L. Elliott, and W. R. Hardy, *J. Amer. Chem. Soc.*, 91, 7528 (1969).

and other experiments (included in this paper) showing that the reaction clearly involves attack of peroxide at the sulfonate of the modified enzyme, we originally proposed that C–O scission to give hydroperoxy enzyme (eq 1), rather than S–O scission to give hydroxy enzyme (eq 2), was taking place. Several lines of approach




were taken directly on the recovered reactivated material. These involved hydroperoxide analysis by *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine oxidation to Wurster's Blue, loss of catalytic activity by $Fe^{2+}$ reduction, and $\beta$-naphthoic acid sensitized photolysis.

This paper describes our work on this system in detail, including a number of additional lines of evidence showing that *the reactivated enzyme is normal ser-195 hydroxy enzyme that has been oxidized by hydrogen peroxide at another position (methionine)*. With the data from the accompanying publication,[4] it is also shown that the hydrogen peroxide–PMS-CT reaction is enzyme catalyzed.

## Experimental Section

**Materials.** Buffer components (dissolved in double distilled water) and standard reagents were all reagent grade commercial products unless otherwise noted. Acetonitrile (Mallinckrodt Nanograde) was distilled from $CaH_2$. *p*-Nitrophenyl acetate, *N-trans*-cinnamoylimidazole (recrystallized from cyclohexane, mp 133–134°), and *N*-acetyl-L-tryptophan ethyl ester (mp 78–79°) were from Mann. L-Tosylphenylalanine chloromethyl ketone (Cyclo), 2-nitro-4-carboxyphenyl *N,N*-diphenylcarbamate (Pierce Chemical Co.), *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (Eastman), and *N*-phenylbenzohydroxamic acid (K & K) were reagent grade. [7-$^{14}$C]Benzyl chloride (0.28 Ci/mol) was from ICN, Polar Brill Blue a gift from Geigy Chemicals, and PAM a gift of Dr. Roy Fukuto of this department.

Methyl hydroperoxide was prepared from hydrogen peroxide and dimethyl sulfate.[5] PMSF[6] and [7-$^{14}$C]PMSF,[7] from benzyl chloride, were synthesized by literature methods. Both labeled and unlabeled PMS-CT were prepared from Worthington 3× recrystallized $\alpha$-chymotrypsin (gel filtered on Sephadex G-25 before use) by the method of Fahrney and Gold.[7] DIP-CT was the Worthington product. $Ti(SO_4)_2$ was prepared from $TiCl_4$ *via* basic hydrolysis to $TiO_2$ followed by treatment with $H_2SO_4$.[8]

**Preparation of Enzyme Derivatives.** A typical preparation of reactivated $\alpha$-chymotrypsin involved adding 100 mg of 0.17% active PMS-CT to a solution containing 5.0 ml of 0.2 *M* phosphate buffer (pH 7.0), 4.25 ml of $H_2O$, and 0.75 ml of 30% $H_2O_2$. The resulting solution, 0.1 *M* in buffer and 0.6 *M* in peroxide, was adjusted to pH 7.0 with 0.01 *M* NaOH. The reaction mixture was allowed to stand for 24–36 hr at 25° (maximum activity) after which it was dialyzed against four changes of 2000 ml of $10^{-3}$ *M* HCl and then against distilled water and lyophilized. No $H_2O_2$ titer was observable after the third buffer change. CT($H_2O_2$) was prepared by adding CT to 0.6 *M* hydrogen peroxide in 0.1 *M* phosphate buffer and allowing it to react for about 24 hr after which it was dialyzed and lyophilized as above.

**Methods.** Hydrogen peroxide was determined by the standard iodimetric method and methyl hydroperoxide by a modification of that procedure using isopropyl alcohol[9] as solvent. Determination of $H_2O_2$ in methyl hydroperoxide was done by using fresh $Ti(SO_4)_2$ solutions and observing visible absorption spectra (a broad peak with $\lambda_{max}$ at 409 nm, $\epsilon$ = 708, was obtained for the $O_2^{2-}$–$Ti(SO_4)_2$ complex)[10,11] with standard $H_2O_2$ solutions. Methyl hydroperoxide, after distillation, contained <0.3% $H_2O_2$, and in no protein–$CH_3OOH$ reaction or control was any observable amount of $H_2O_2$ generated.

Catalytic rates and active site titrations were performed by standard methods.[12–15] Reactivations were followed by removing appropriate size aliquots from enzyme–reagent incubations and adding to a cuvette containing assay or titration mixture in a Cary 14 spectrophotometer.

Inhibitions were examined by literature procedures. For TPCK, to 6.5 mg of react-CT (or 3.7 mg of native CT) in 20 ml of pH 7.0 phosphate buffer (0.1 *M*) was added TPCK (7 mg) in 0.5 ml of methanol. Activity was monitored with ATrEE.

Loss of label from [7-$^{14}$C]PMS-CT was followed by dialyzing aliquots from reaction mixtures *vs.* $10^{-3}$ *M* HCl and then counting in 10 ml of cocktail (made from 2,5-diphenyloxazole (7 g), 1,4-bis(2-(4-methyl-5-phenyl)oxazolyl)benzene (0.3 g), and naphthalene (10 g) in 1.0 l. of dioxane solution), analyzing for protein (taking $\epsilon_{282}$ = $5.15 \times 10^4$ $M^{-1}$ cm$^{-1}$),[16] and assaying for catalytic activity and/or active sites. In a typical run 75 mg of [7-$^{14}$C]PMS-CT ($2.7 \times 10^8$ cpm mol$^{-1}$) was added to 7.0 ml of buffer, 0.55 ml of 30% $H_2O_2$ was added, and the pH was adjusted to the desired value.

Amino acid analysis was by standard methods on a Beckman Model 120C,[17] and end groups were determined by Stark's method.[18]

**Isotope Dilution Measurement of Sulfonic Acid Liberated.** To [7-$^{14}$C]PMS-CT (990 mg) was added 25 ml of phosphate buffer (0.2 *M*, pH 7.0) and 20 ml of $H_2O$. $H_2O_2$ (3.75 ml, 30%) was added and the pH was adjusted to 7.0 with NaOH. Before the $H_2O_2$ was added the protein (uv) concentration was determined, two 100-$\lambda$ samples were counted for $^{14}$C label, and the initial rate of 50 $\lambda$ of the solution was determined by an assay with ATrEE (there was less than 0.5% catalytic activity). After 65 hr at room temperature the protein was precipitated with 4 ml of concentrated HCl and centrifuged. To the resulting protein-free solution was added 920 mg of the sodium salt of phenylmethanesulfonic acid. This solution was then lyophilized and suspended in $CHCl_3$ (75 ml), and 6 g of $PCl_5$ was added over a period of 15 min. The $CHCl_3$ was washed with $H_2O$, dried with $MgSO_4$, and evaporated. PMSCl (18.5 mg) was recovered and recrystallized several times from benzene–cyclohexane. (Counting data are given in Table VII.)

## Results

**General Description of the Reaction.** In all experiments PMS-CT, produced from the sulfonyl fluoride (PMSF) and gel-filtered chymotrypsin (CT), initially was <1% active toward methyl or ethyl *N*-acetyl-L-tryptophanate (ATrME or ATrEE) and had <1% active sites in titrations with cinnamoylimidazole. Figure 1, a typical reactivation, shows that in approximately 12–15 hr with 0.6 *M* hydrogen peroxide at pH 7, a maximum of ~50% catalytic ($S_0 \gg K_m$) activity toward ATrEE is obtained while PMS-CT in buffer alone shows no effect and CT in hydrogen peroxide has lost approximately 10% of its catalytic activity. In our work, as in the original work of Fahrney and Gold,[7] no reactivation occurs in buffer alone over very extended time periods except at low pH (nor is there loss of $^{14}$C

(4) N. A. Radomsky and M. J. Gibian, *J. Amer. Chem. Soc.*, **95**, 8713 (1973).
(5) A. Rieche and F. Hitz, *Ber.*, **62**, 2458 (1929).
(6) C. W. Tullock and D. D. Coffman, *J. Org. Chem.*, **25**, 2016 (1960); T. B. Johnson and J. M. Sprague, *J. Amer. Chem. Soc.*, **58**, 1348 (1936).
(7) (a) D. Fahrney and A. M. Gold, *ibid.*, **85**, 997 (1963); (b) A. M. Gold and D. Fahrney, *Biochemistry*, **3**, 783 (1964); **5**, 2911 (1966).
(8) C. W. Wood and A. K. Holliday, "Inorganic Chemistry," 3rd ed, Plenum Press, New York, N. Y., 1967, p 319.
(9) C. D. Wagner, R. H. Smith, and E. D. Peters, *Anal. Chem.*, **19**, 979 (1947).
(10) J. A. Connor and E. A. V. Ebsworth, *Advan. Inorg. Chem. Radiochem.*, **6**, 279 (1964).
(11) R. J. H. Clark, "The Chemistry of Titanium and Vanadium," Elsevier, New York, N. Y., 1968, p 210.
(12) M. L. Bender, G. E. Clement, F. J. Kézdy, and H. d'A. Heck, *J. Amer. Chem. Soc.*, **86**, 3680 (1964).
(13) G. Schonbaum, B. Zerner, and M. L. Bender, *J. Biol. Chem.*, **236**, 2930 (1961); M. L. Bender, *et al.*, *J. Amer. Chem. Soc.*, **88**, 5890 (1966).
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(15) B. Erlanger and F. Edel, *Biochemistry*, **3**, 346 (1964).
(16) G. W. Schwert and S. Kaufman, *J. Biol. Chem.*, **190**, 807 (1951).
(17) N. P. Neuman, *Methods Enzymol.*, 11, 485, 487 (1967).
(18) G. Stark, *ibid.*, 11, 125 (1967).

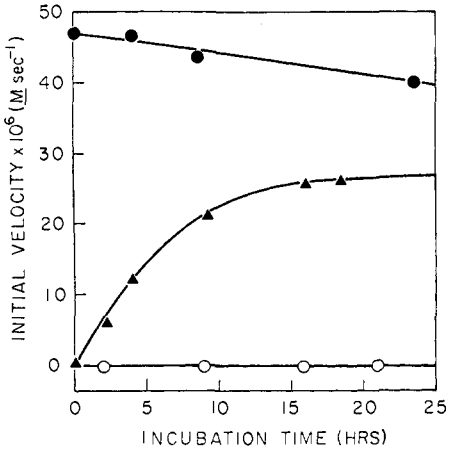



Figure 1. Effect of hydrogen peroxide (0.60 *M*) on α-chymotrypsin (●) and PMS-CT (▲) and buffer alone on PMS-CT (○) *vs.* time. These runs were all incubated at $3 \times 10^{-4}$ *M* protein in 0.1 *M* phosphate buffer, pH 7.0, 25°. Assays were as described in the Experimental Section toward ethyl *N*-acetyl-L-tryptophanate.

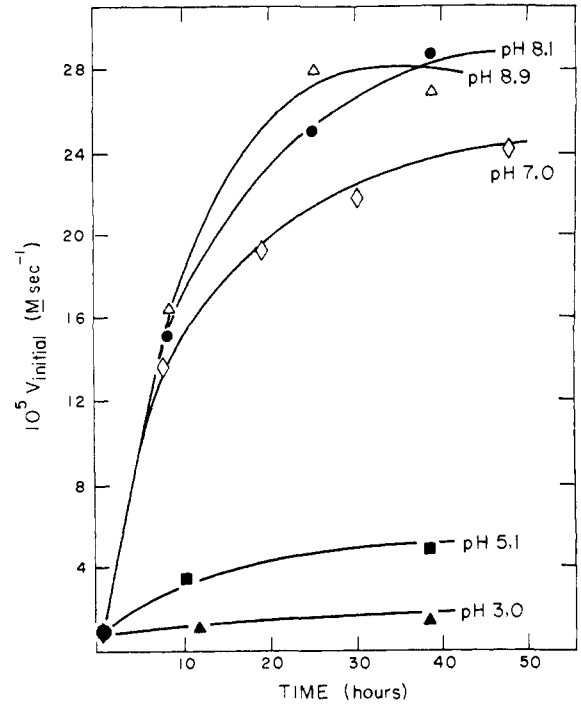



Figure 2. Activity *vs.* time of aliquots of incubation mixtures of PMS-CT allowed to react with 0.6 *M* hydrogen peroxide in 0.1 *M* phosphate (pH 5.1 and 7.0), 0.1 *M* Tris (pH 8.1 and 8.9), and $10^{-3}$ *M* HCl. The initial velocities were toward ATrEE, standard conditions, but the values are adjusted to 0.5 mg of protein in the assay cell (aliquots were dialyzed prior to assay).

from the radioactive derivative, *vide infra*). Figure 2 shows reactivation rates for several pH values; above pH 9 competing denaturation of the enzyme effectively prohibits meaningful experiments. The reaction at pH 7.0, 0.1 *M* phosphate buffer, and 0.6 *M* hydrogen peroxide was used to maximize the yield of reactivated material. Reactivated enzyme was isolated as described in the Experimental Section, and this dialyzed and lyophilized enzyme was that usually used, except in a few cases in which the solution obtained subsequent to dialysis but preceding lyophilization was examined *vs.* the lyophilized product. In each instance no discernibly different result was observed. Total protein concentration for a given solution was determined by comparing $A_{282}$ with weight concentrations for a given preparation.

**Properties of Reactivated Enzyme.** A series of active site determinations was performed using *N-trans*-cinnamoylimidazole (CI),[13] *p*-nitrophenyl acetate (NPA),[14] and 2-nitro-4-carboxyphenyl *N,N*-diphenylcarbamate (NCDC)[15] in standard procedures with starting α-chymotrypsin, reactivated chymotrypsin, and α-chymotrypsin that itself had been treated with hydrogen peroxide, dialyzed, and lyophilized in a procedure identical with that used on PMS-CT in order to reactivate it [subsequently referred to as $CT(H_2O_2)$]. These data, which measure active site concentrations directly from bursts, are shown in Table I. In the standard NCDC procedure for CT, a precipitate (suggested by Erlanger[15] to be the calcium salt of NCDC) is formed which then redissolves. For $CT(H_2O_2)$ the precipitate remained for an extended period but eventually did dissolve. A low titer was found. For react-CT, precipitate never redissolved (45 min), and no titer at all was obtained. The NPA and CI titrations show agreement on the concentration of active sites for all three proteins.

**Table I.** Active Site Titrations on Modified Enzyme[a]

| | CT × $10^4$, *M* | React-CT × $10^4$, *M* | $CT(H_2O_2)$ × $10^4$, *M* |
|---|---|---|---|
| NPA | 2.94 | 1.74 | 3.62 |
| CI | 2.85 | 1.64 | 3.72 |
| NCDC | 2.81 | (0) | (1.65) |

[a] Standard assay conditions, see references. Each enzyme was at a different concentration so that only comparison down a column is meaningful.

Table II gives the pH dependence of the kinetic parameters of catalysis of ATrEE hydrolysis by each of the three enzyme preparations. The data for α-CT itself are in quite reasonable agreement with the commonly observed behavior of this system.[12] It may be seen that the values for $CT(H_2O_2)$ and react-CT are in agreement with each other, giving $k_{cat}$(max) about twice that for CT and a slightly shifted $k_{cat}/K_m$ dependence at high pH. The elevated $k_{cat}$ had previously been observed for met-192 sulfoxide-CT,[19] and in our earlier work we had not compared react-CT to $CT(H_2O_2)$, leading to a conclusion different from that which we now see as inescapable.

While each of the methods mentioned in the introduction for determining whether react-CT has a peroxide group showed positive results with react-CT compared to CT (Wurster's Blue formation and fivefold faster inactivations by the latter two tests), $CT(H_2O_2)$ as a control in each test shows the same result as react-CT. The dialysis procedure used to free both $CT(H_2O_2)$ and react-CT from hydrogen peroxide results in no hydrogen peroxide left inside the membrane in the absence of protein, so the results of these analyses are likely due to the hydrogen peroxide changed protein.[20]

(19) H. Weiner, C. W. Blatt, and D. E. Koshland, Jr., *J. Biol. Chem.*, 241, 2687 (1966).

(20) It could be that hydrogen peroxide is specifically entrained by protein, but the length of dialysis (~24 hr) exceeds the length of hydrogen peroxide incubation, and it is unlikely that a purely nonchemical trapping would be highly specific for hydrogen peroxide *vs.* $H_2O$ at pH 7.

**Table II.** Catalytic Behavior of Native, Reactivated, and Hydrogen Peroxide Treated Chymotrypsin toward Ethyl *N*-Acetyl-L-tryptophanate[a]

| | | $k_{cat}$, $sec^{-1}$ | | | $10^5 K_m$, $M$ | | | $10^5(k_{cat}/K_m)$, $M^{-1}$ $sec^{-1}$ | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | CT | React-CT | $CT(H_2O_2)$ | CT | React-CT | $CT(H_2O_2)$ | CT | React-CT | $CT(H_2O_2)$ |
| 5.80 | P[b] | 3.0 ± 0.3 | 7.9 ± 0.6 | | 23.5 ± 6.0 | 40.3 ± 6.0 | | 0.13 ± 0.05 | 0.20 ± 0.05 | |
| 6.36 | P | 15.2 ± 0.4 | 27.8 ± 1.0 | | 11.7 ± 1.0 | 30.0 ± 1.0 | | 1.3 ± 0.2 | 0.93 ± 0.06 | |
| 6.51 | P | | | 29.0 ± 2.0 | | | 26.0 ± 5.0 | | | 1.1 ± 0.3 |
| 6.67 | P | 19.3 ± 1.0 | 39.0 ± 2.0 | | 11.0 ± 1.0 | 27.0 ± 3.0 | | 1.8 ± 0.2 | 1.4 ± 0.3 | |
| 7.07 | P | 28.9 ± 1.0 | 55.6 ± 1.0 | 58.0 ± 1.0 | 10.6 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 2.0 | 2.7 ± 0.4 | 2.0 ± 0.1 | 2.1 ± 0.2 |
| 7.32 | T | | | 82.0 ± 3.0 | | | 37.0 ± 3.0 | | | 2.2 ± 0.3 |
| 7.52 | T | 39.6 ± 4.0 | 60.5 ± 6.0 | | 11.9 ± 2.0 | 20.2 ± 3.0 | | 3.3 ± 1.1 | 3.0 ± 0.9 | |
| 7.63 | P | 43.0 ± 2.0 | 73.0 ± 1.0 | | 12.3 ± 3.0 | 26.0 ± 1.0 | | 3.6 ± 1.2 | 2.8 ± 0.2 | |
| 7.75 | P | | | 104 ± 9 | | | 30.0 ± 5.0 | | | 3.5 ± 1.0 |
| 7.85 | T | 39.8 ± 1.0 | | | 12.7 ± 1.0 | | | 3.1 ± 0.4 | | |
| 8.09 | T | | 94.0 ± 5.0 | | | 30.0 ± 3.0 | | | 3.1 ± 0.6 | |
| 8.15 | T | 48 ± 1 | 99.0 ± 6.0 | 93.0 ± 3 | 17 ± 1 | 33.0 ± 4.0 | 36.0 ± 2.0 | | 3.0 ± 0.6 | 2.6 ± 0.3 |
| 8.29 | T | 39.5 ± 3.0 | | | 16.8 ± 4.0 | | | 2.4 ± 0.4 | | |
| 8.42 | T | | 94.0 ± 5.0 | | | 26.2 ± 4.0 | | | 3.6 ± 0.9 | |
| 8.68 | C | 41.8 ± 4.0 | 81.0 ± 2.0 | | 16.5 ± 3.0 | 22.8 ± 1.0 | | 2.5 ± 0.8 | 3.7 ± 0.1 | |
| 8.81 | C | 39.5 ± 0.5 | 88.0 ± 5.0 | | 22.7 ± 1.0 | 20.0 ± 3.0 | | 1.7 ± 0.1 | 4.4 ± 1.0 | |
| 8.90 | T | | | 92.0 ± 9.0 | | | 42.0 ± 5.0 | | | 2.2 ± 0.5 |
| 8.95 | C | 48.8 ± 3.0 | | | 27.2 ± 4.0 | | | 1.8 ± 0.4 | | |
| 9.36 | C | | 83.4 ± 4.0 | | | 32.3 ± 6.0 | | | 2.6 ± 0.7 | |
| 9.69 | C | 59.0 ± 1.0 | 101 ± 5 | | 86.0 ± 5.0 | 55.0 ± 8.0 | | 0.69 ± 0.03 | 1.8 ± 0.5 | |
| 9.92 | C | 44.0 ± 2.0 | 98.0 ± 1.0 | | 102 ± 15 | 64.0 ± 4.0 | | 0.43 ± 0.10 | 1.5 ± 0.1 | |

[a] $S_0$ was initially about $5\text{–}8K_m$ in each run and $E_0$ about $10^{-7}$ $M$ (titrated by cinnamoylimidazole). Reaction was followed to $S \simeq 0.5K_m$. [b] P = phosphate, T = Tris, C = carbonate. All 0.1 $M$ buffers.

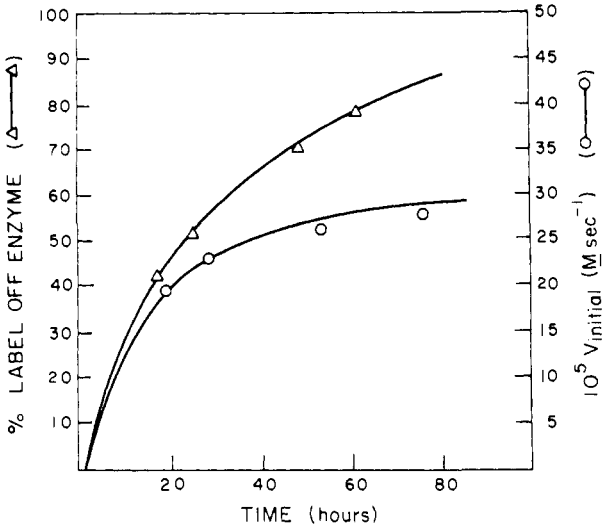



Figure 3. Catalytic activity (toward ATrEE) and $^{14}C$ loss of [7-$^{14}$C]PMS-CT *vs.* time incubated with 0.6 $M$ $H_2O_2$ in 0.1 $M$ phosphate buffer, pH 7.0, 25°.

These peroxide analysis results are thus not definitive for either eq 1 or 2.

The reactivated material is readily covalently inhibited by PMSF under the usual conditions, and the resultant protein can only be reactivated by hydrogen peroxide (see below). React-CT is slowly inhibited by TPCK[21] compared to native CT (respective pseudo-first-order rate constants of $0.4 \times 10^{-4}$ *vs.* $1.4 \times 10^{-4}$ $sec^{-1}$ for $7 \times 10^{-6}$ $M$ protein, $10^{-4}$ $M$ TPCK, 0.1 $M$ phosphate buffer, pH 7.0).[22] CT alkylated at met-192, which has similar catalytic activity to enzyme oxidized at this position, has been reported to react with TPCK about 30 times slower than does CT,[23] so that our result is not unexpected.

(21) E. B. Ong, E. Shaw, and G. Schoellmann, *J. Biol. Chem.*, **240**, 694 (1965).
(22) F. J. Kézdy, A. Thomson, and M. L. Bender, *J. Amer. Chem. Soc.*, **89**, 1004 (1967).
(23) W. B. Lawson and H. J. Schramm, *Biochemistry*, **4**, 377 (1965).

α-Chymotrypsin has two methionine residues, one (met-192) being considerably more susceptible to oxidation (by $H_2O_2$ at pH 3 and by NaOI) than the other, the oxidation of which causes only small changes in activity.[19,24,25] By first incubating with iodoacetate to alkylate all free methionine, then oxidizing with performic acid to convert methionine sulfoxide to the sulfone, and then performing an acid hydrolysis, it was possible to test by amino acid analysis for the state of the methionines in native, reactivated, and $H_2O_2$-incubated CT.[17,18] Within experimental error the only amino acid affected upon $H_2O_2$ treatment was methionine, one of the two residues having been oxidized (for methionine sulfone, CT gives 0.2 ± 0.05, react-CT gives 1.3 ± 0.06, and $CT(H_2O_2)$ gives 0.95 ± 0.01). Attempts at end group analysis using cyanogen bromide on react-CT and $CT(H_2O_2)$ were unsuccessful but are actually not necessary for unambiguous assignment of met-192 as the sole oxidized moiety in both proteins (see Discussion).

**The Reactivation Reaction with Hydrogen Peroxide.** The data of Figure 2 were actually obtained in a series of experiments in which loss of radioactive label from [7-$^{14}$C]PMS-CT and catalytic activity toward ATrEE were monitored during the incubation with $H_2O_2$ at pH values from 3.0 to 8.9. At each pH the initial rate of $^{14}C$ loss closely correlated with catalytic activity regain, a typical run being shown in Figure 3. The two curves diverge after about 50% reaction because of subsequent denaturation of react-CT (controls using $CT(H_2O_2)$ show the same effect, Figure 1). Indeed, as shown in Figure 4, the $^{14}C$ rate is first order for at least 75% of the reaction. Figure 4 also shows the first-order behavior for all runs from pH 3 to 8.9, and in Figure 5 the rate constants from Figure 4 are plotted against pH.

Data for the run at pH 7, which included active site titrations with cinnamoylimidazole, are given in Table

(24) H. Schachter and G. Dixon, *J. Biol. Chem.*, **239**, 813 (1964).
(25) J. Knowles, *Biochem. J.*, **95**, 180 (1965).

**Table III.** Stoichiometry of $^{14}C$ Loss and Appearance of Active Sites

| A<br>Time, hr | B[a]<br>Active sites/ uv concn (for CT + $H_2O_2$) | C[a]<br>Active sites/ uv concn (for PMS-CT + $H_2O_2$) | D[b]<br>$10^{-8}$ cpm/mol of protein (by uv) | E[c]<br>Fraction of $^{14}C$ off enzyme | F[d]<br>Predicted fraction of active sites for react-CT |
|---|---|---|---|---|---|
| 0 | 1.00 | | 2.45 | | |
| 19 | 0.94 ± 0.06 | 0.29 ± 0.07 | 1.44 | 0.41 | 0.38 ± 0.03 |
| 30 | 0.80 ± 0.02 | 0.42 ± 0.05 | 0.995 | 0.595 | 0.47 ± 0.01 |
| 48 | 0.72 ± 0.04 | 0.52 ± 0.04 | 0.70 | 0.715 | 0.52 ± 0.03 |
| 66 | 0.73 ± 0.05 | 0.59 ± 0.02 | 0.48 | 0.805 | 0.59 ± 0.04 |
| 94 | 0.74 ± 0.08 | 0.78 ± 0.10 | 0.25 | 0.90 | 0.67 ± 0.07 |
| 162 | 0.74 ± 0.08 | 0.86 ± 0.13 | (0.30)[e] | (0.88)[e] | (0.65 ± 0.07)[e] |

[a] 0.6 *M* $H_2O_2$ and CT (or PMS-CT) in 0.1 *M* phosphate buffer, pH 7.0. Aliquots dialyzed *vs.* four changes of distilled water. Duplicate CI titrations for active sites and protein uv absorbance at 282 nm were done. [b] 500 λ of dialyzed solution was counted for $^{14}C$. The cpm were corrected by using $A_{282}$ and assuming mol wt = 25,000. [c] 1.00 − ([cpm/mol(time *t*)]/2.45 × $10^{-8}$); *i.e.*, 1.00 − fraction of $^{14}C$ lost. [d] Column B × column E. If each $^{14}C$ loss gives one site, and loss of catalytic activity caused by $H_2O_2$ side reactions is the same for CT and PMS-CT, column F should equal column C. [e] This last point is quite inaccurate because of dilution of protein. In other runs it was found that at long incubation times >95% of the label is lost.

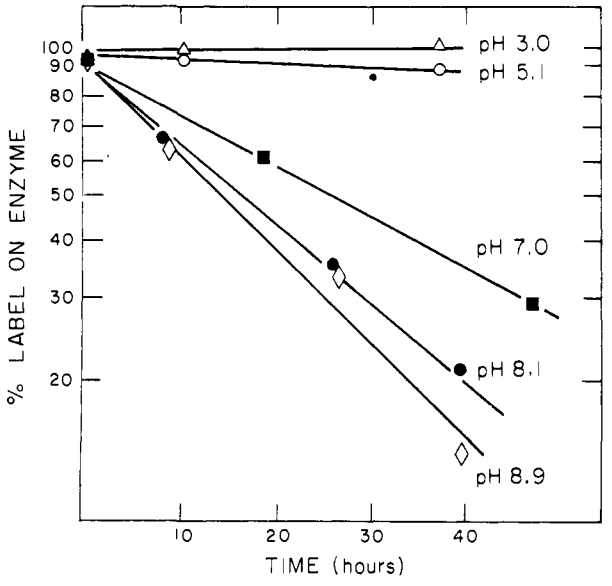



Figure 4. First-order plots of $^{14}C$ loss for runs of Figure 2.

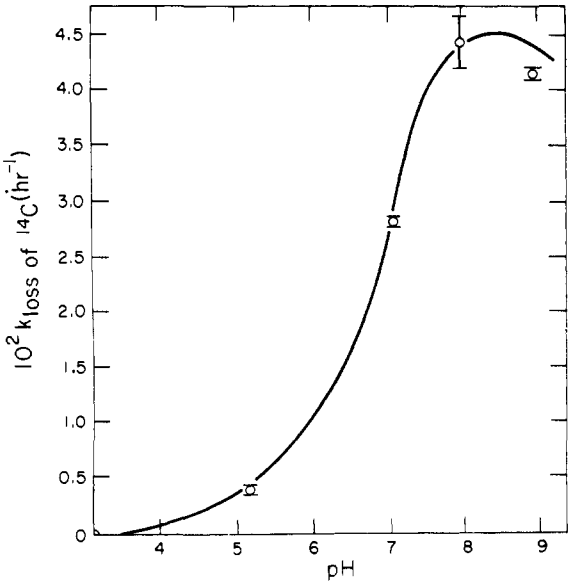



Figure 5. Dependence of rate constants of Figure 4 upon pH.

III along with a control run using CT plus $H_2O_2$. A sample from the same preparation of [7-$^{14}C$]PMS-CT used for these runs was subjected to desulfonation at 40° and pH 3, and all $^{14}C$ was removed in a clean first-order process in complete agreement with Fahrney and Gold.[7] Columns C and D give active sites and $^{14}C$ remaining as the incubation proceeds; column B, a control of native enzyme with $H_2O_2$, indicates the slow destruction of active sites. Column E, the fraction of $^{14}C$ lost at the given times (derived from column D), is then corrected for loss of activity by incubation as observed for CT itself (column B) to give the last column of data. Thus, column F predicts the number of active sites from $^{14}C$ loss and independent destruction data and should be compared to column C, the per cent of active sites experimentally determined. There is excellent quantitative agreement to 90% of the reaction, and we conclude that *each site is regained concommitant with the loss of one* $^{14}C$, and hence one sulfonate.

Table IV shows the results of an experiment in which CT($H_2O_2$) was produced and then subjected to the series of reactions and controls usually used on normal CT in this work. CT($H_2O_2$), as reported above, is rapidly inhibited by PMSF to give PMS-CT($H_2O_2$). This latter preparation is totally unreactive in buffer alone but is reactivated by hydrogen peroxide. Thus, although hydrogen peroxide produces a modified CT, this material is readily sulfonated to give a derivative that is stable until hydrogen peroxide treatment, showing that in the reactivation *hydrogen peroxide itself must react with the* $-CH_2SO_2OCH_2R$ *and cannot be solely reacting at another site of the protein that serves to labilize sulfonate.*

**Table IV.** Effect of $H_2O_2$ on α-CT Reactions

| Sample | $10^5 V_{in}$, *M* $sec^{-1}$ |
|---|---|
| α-CT | 7.46 |
| $H_2O_2$, 22 hr | 6.12 |
| Dialyze (dilution) | 5.29 |
| PMSF, dialyze | 0.13 |
| $H_2O_2$, 3 hr | 0.30 |
| $H_2O_2$, 24 hr | 1.03[a] |
| $H_2O$, 3 hr | 0.13 |
| $H_2O$, 24 hr | 0.13 |

[a] Correcting for dilutions, this is 30% net reactivation, about normal for the usual experiments.

In Table V and Figure 6, react-CT and CT, side by side, were both rapidly inhibited by PMSF and the two

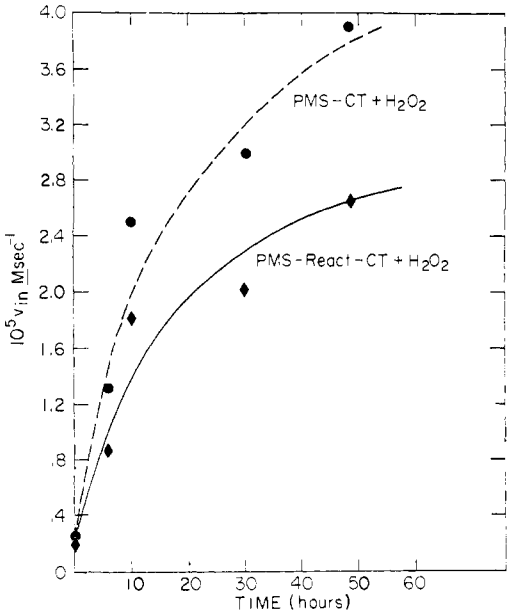



Figure 6. Reactivation (measured by ATrEE assay) of PMS-CT and PMS-react-CT (10 mg/ml in incubation solution) at pH 7.0, 0.1 *M* phosphate buffer with 0.6 *M* $H_2O_2$. Original CT and react-CT both had $1.4 \times 10^{-5}$ *M* sec$^{-1}$ activities; after 15 min with PMSF the respective activities were $6.9 \times 10^{-8}$ *M* sec$^{-1}$ and $4.0 \times 10^{-8}$ *M* sec$^{-1}$. They were then dialyzed and used in the experiment shown here.

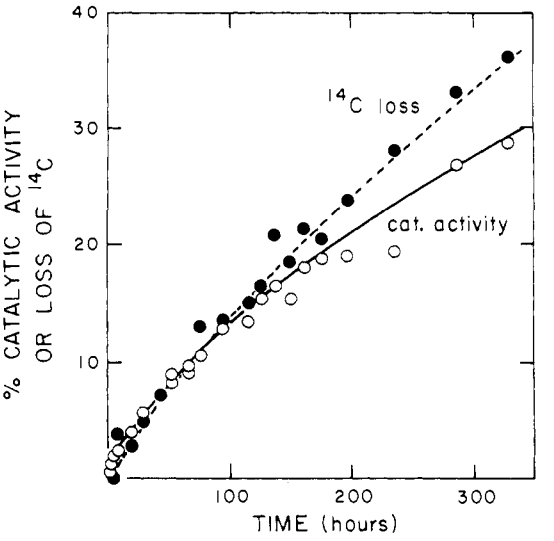



Figure 7. Same as Figure 3 but for 0.5 *M* methyl hydroperoxide.

**Table V.** Reaction of PMS-CT and PMS-React-CT with Hydrogen Peroxide[a,b]

| Time, hr | $10^5 V_{in}$, *M* sec$^{-1}$ PMS-CT | $10^5 V_{in}$, *M* sec$^{-1}$ PMS-React-CT |
|---|---|---|
| 0 | 0.33 | 0.32 |
| 6 | 1.33 | 0.86 |
| 10 | 2.5 | 1.8 |
| 30 | 3.0 | 2.0 |
| 48 | 3.8 | 2.6 |

[a] Reactions with PMSF as described before, then each sample was dialyzed, lyophilized, and redissolved as described below. [b] Conditions are: 0.5 ml of 0.2 *M* (total) phosphate buffer pH 7.0 + 0.075 ml of 30% $H_2O_2$ (0.66 *M*) + 2 drops of NaOH + 10 mg of the modified enzyme in 0.5 ml of water. Assays were done toward ATrEE in the usual manner.

modified samples were subjected to both hydrogen peroxide treatment and buffer incubation. The two samples behaved almost identically. It is possible, but very unlikely, that a peroxy sulfonate would behave in this way.[26]

**Reactions with Various Nucleophilic Reagents.** Other nucleophiles were tried with PMS-CT, the first of which were alkyl hydroperoxides. Reasoning that nucleophilic attack by $ROO^-$ should be very similar to that by $HOO^-$, *n*-propyl hydroperoxide (0.2 *M*) was incubated with PMS-CT and aliquots were tested for catalytic activity for ~24 hr.[3] During this time hydrogen peroxide had produced a 100-fold increase in activity of the sample (up to 50% on a weight basis); $n$-$C_3H_7O_2H$ produced only a slight increase. In our earlier report, this slight increase was assumed to be due to small amounts of hydrogen peroxide impurity and the general result taken as evidence for nucleophilic attack at carbon rather than sulfur, since the former process would produce an inactive E–$CH_2OOR$ while the latter mode would produce E–$CH_2OH$ and $C_6H_5CH_2SO_2OOR$. In subsequent experiments we used $CH_3OOH$ because of its less deleterious effect on enzyme at 0.5 *M* ($\alpha$-CT with 0.5 *M* $n$-$C_3H_7OOH$ becomes cloudy).

As described in the Experimental Section, the $CH_3OOH$ (using $Ti(SO_4)_2$) proved to be completely free of hydrogen peroxide (<0.3%). The reaction mixture with protein and a control of $CH_3OOH$ with no protein but kept under the conditions of the protein experiments showed no production of hydrogen peroxide over the time scale of the experiments. In agreement with the earlier $n$-$C_3H_7OOH$ observation, in 24 hr $CH_3OOH$ produced a small, but significant, reactivation. Over very extended time periods, however (about 2 weeks), the reactivation was about as much as that caused by hydrogen peroxide. Labeled PMS-CT was used in an experiment with $CH_3OOH$ in which catalytic activity, $^{14}C$ loss, and CI titratable active sites *vs.* $A_{282}$ protein concentrations were recorded on aliquots taken at various times (Figure 7). The CI titrations were very difficult because of the low values, dilution of the protein, and necessarily rather small quantities available. The latest times are fairly good, though. As seen in Figure 7 the results show a parallel loss of $^{14}C$ and reactivation with no significant time lag between them, but at rates much lower than for hydrogen peroxide.

Figure 8 shows rate assays to test for reactivation of PMS-CT with $H_2O_2$, $C_6H_5CON(C_6H_5)OH$, $C_6H_5OH$, $N_2H_4$, and PAM. None of these except $H_2O_2$ caused reactivation. Figure 9 shows similar experiments for PMS-CT as well as for diisopropylphosphoryl-chymotrypsin (DIP-CT) with $H_2O_2$ and $NH_2OH$. Hydroxylamine appears to be very slightly effective in causing reactivation of PMS-CT, and DIP-CT is very slightly (about equally) affected by $H_2O_2$ and $NH_2OH$. To further investigate the PMS-CT reaction with $NH_2OH$, [7-$^{14}C$]PMS-CT was incubated with 0.5 *M* $NH_2OH$ and rate assays as well as loss of $^{14}C$ were followed for 7

(26) In contrast to the general stability of hydroperoxides, peroxy esters (and especially persulfonates) are rather unstable to a wide variety of conditions: "Organic Peroxides," D. Swern, Ed., Wiley-Interscience, New York, N. Y., 1970.

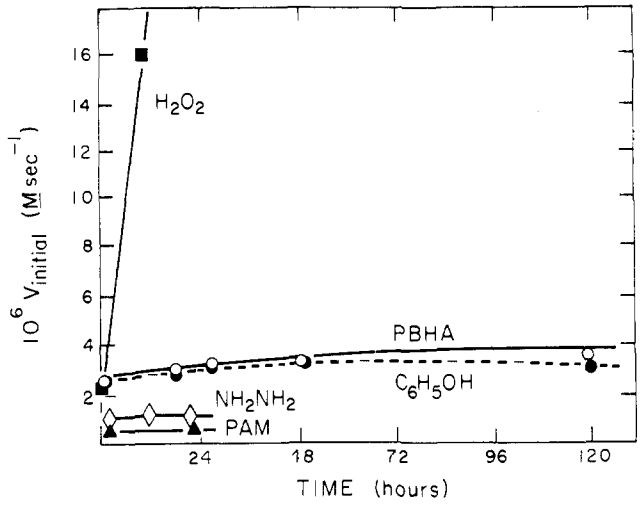



Figure 8. Catalytic activity (toward ATrEE, usual conditions) of aliquots of PMS-CT ($2 \times 10^{-4}$ $M$ in reaction mixture) incubated with various nucleophiles in 0.1 $M$ phosphate buffer, pH 7.0, 25°: (■) 0.6 $M$ $H_2O_2$; (○) 0.0025 $M$ $N$-phenylbenzohydroxamic acid; (●) 0.01 $M$ phenol; (◇) 2.5 $M$ hydrazine; (▲) 0.05 $M$ pyridine-2-aldoxime methiodide.

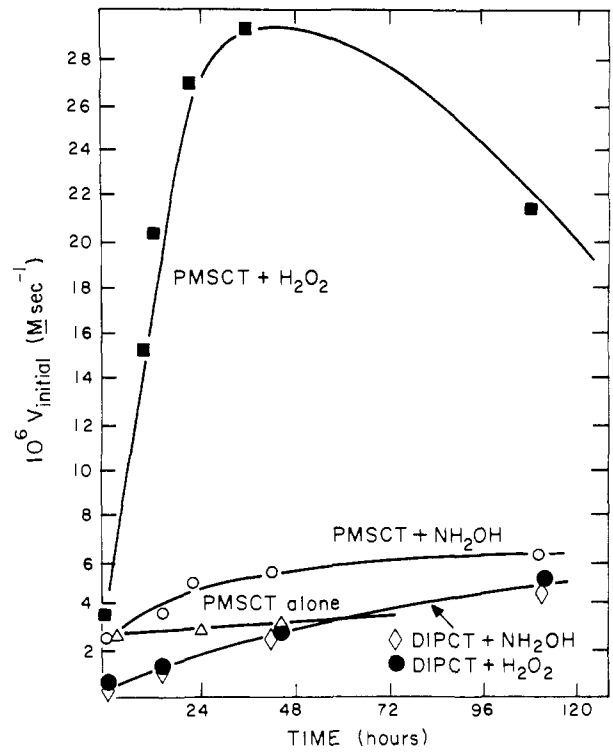



Figure 9. Catalytic activity (toward ATrEE, usual conditions) of aliquots of PMS-CT and DIP-CT ($2 \times 10^{-4}$ $M$ in reaction mixture) incubated with nucleophiles in 0.1 $M$ phosphate buffer, pH 7.0, 25°: (■) 0.5 $M$ $H_2O_2$ + PMS-CT; (●) 0.5 $M$ $H_2O_2$ + DIP-CT; (○) 0.5 $M$ hydroxylamine + PMS-CT; (◇) 0.5 $M$ hydroxylamine + DIP-CT; (△) PMS-CT, no added reagent.

days. At that time only 4% of the label was lost, and very little activity was regained.[27]

**Fate of the Sulfonate Moiety.** The question of S–O *vs.* C–O scission was approached by analysis for the sulfonate product. The two possibilities (see Discussion) are phenylmethanepersulfonic acid (eq 2) or phenylmethanesulfonic acid (eq 1) resulting from the respective sites of $H_2O_2$ attack. The persulfonic acid is most likely very unstable,[26] and a method that traps persulfonate very rapidly, involving oxidation of the dye Polar Brill Blue, was utilized.[28] This material, which absorbs at 620 nm, is quantitatively and very rapidly oxidatively bleached by persulfonates but is inert (at least on the time scales involved here) toward $H_2O_2$, $HO_2^-$, and alkyl hydroperoxides. The first entries in Table VI show the effect of adding excess $H_2O_2$ to methanesulfonyl chloride (this generates methanepersulfonic acid) in the presence of Polar Blue. The reaction is complete in 10 min, and $\Delta A$ is linear in the concentration of sulfonyl chloride, and thus in persulfonate. The PMS-CT reaction with $H_2O_2$ in the presence of dye was allowed to proceed for 20 hr, enough to generate about 50% of the product, and no titer was observed for persulfonate as seen in the second set of entries of Table VI. The last two sets of data in this table are controls. It seems that no free persulfonate is formed in the PMS-CT reaction with $H_2O_2$. It is possible that persulfonate formed at the active site reacts before it escapes from the enzyme. The $^{14}C$ recovery after dialysis argues against any attachment to the enzyme, but an oxidation of protein may have occurred.

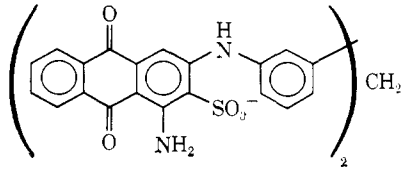
Table VI. Analysis for Persulfonic Acid Formation in Reactions with Hydrogen Peroxide

| Compd | Concn, $10^{-4}$ $M$ | $\Delta A_{620\ nm}$ |
|---|---|---|
| $CH_3SO_2Cl$[a] | 2.6 | 0.17 |
| $CH_3SO_2Cl$ | 6.5 | 0.57 |
| $CH_3SO_2Cl$ | 6.5 | 0.52 |
| PMS-CT[b] | 6.7 | 0.00 |
| PMS-CT | 9.0 | 0.03 |
| CT($H_2O_2$)[c] | 6.7 | 0.00 |
| CT($H_2O_2$) | 9.0 | 0.05 |
| Control[d] | | 0.04 |
| Control | | 0.09 |

[a] Polar Brill Blue was $8 \times 10^{-5}$ $M$ in 0.02 $M$ phosphate, pH 7, buffer containing 0.6 $M$ $H_2O_2$. To this solution was added varying concentrations of methanesulfonyl chloride. The absorbance was read at 620 nm before addition and 10 min later. [b] Same conditions except that the absorbance was determined after 20 hr of reaction (because of the much slower reaction of PMS-CT and $H_2O_2$). The activity of the enzyme before the addition of dye and $H_2O_2$ was less than 1%. After 20 hr of reaction the enzyme showed 25% activity on a protein basis. [c] This was done as in footnote *b*. [d] Control consisted of only the dye and peroxide solution. The absorbance was determined after 20 hr.

(27) Dr. Yechiel Shalitin (personal communication) has made similar observations to these and is in agreement with our results.

(28) This titrant was suggested to us by Dr. Eugene Zeffren, to whom we are very grateful. Its structure is



In fact, by an isotope dilution experiment it was shown that phenylmethanesulfonic acid is formed quantitatively. A known weight of unlabeled sodium phenylmethanesulfonate was added to a reacted solution of [7-$^{14}C$]PMS-CT from which protein had been removed and the supernatant evaporated to dryness. The residue was dissolved in $CHCl_3$ and allowed to react with $PCl_5$, converting sulfonate to sulfonyl chloride. Radioactivity was monitored at each stage, and

**Table VII.** Isotope Dilution Experiment for Phenyl Methanesulfonate

| | |
|---|---|
| Moles of potentially labeled phenyl methanesulfonate sodium salt[a] | $3.17 \times 10^{-5}$ mol |
| Moles of unlabeled phenyl methanesulfonate sodium salt added | $4.75 \times 10^{-3}$ mol |
| Specific activity of PMSCl isolated from the reaction | $1.09 \times 10^{6}$ cpm/mol |
| Specific activity of [7-$^{14}$C]PMS-CT (before the reaction) | $1.62 \times 10^{8}$ cpm/mol |
| Mole fraction of sulfonate salt from original reaction (entry 1/entry 2) | $0.668 \times 10^{-2}$ mol |
| Mole fraction of $^{14}$C recovered (entry 3/entry 4) | $0.675 \times 10^{-2}$ mol |

[a] Calculated by assuming that all $^{14}$C label was displaced in the reaction as the sulfonate salt. The reaction lasted for 60 hr, and previous experiments showed that by that length of time 80% of the $^{14}$C label was removed. Thus, the moles of potentially labeled sulfonate salt were equal to 0.8 times the moles of [7-$^{14}$C]PMS-CT ($3.96 \times 10^{-5}$) in the reaction.

the recovered PMSCl recrystallized several times from benzene–cyclohexane. Table VII shows the data for this experiment and for controls and indicates quantitative formation of $C_6H_5CH_2SO_2O^-$ from the PMS-CT plus $H_2O_2$ reaction.

## Discussion

The rapid and relatively specific reactivation of ser-195 PMS-CT by $H_2O_2$ ultimately produces phenylmethanesulfonic acid and monosulfoxide-chymotrypsin with no other changed groups. For the sulfonic acid, this conclusion arises from quantitative determination of sulfonic acid and lack of observation of persulfonic acid. For the enzyme, a wide variety of results leads to elimination of peroxy enzyme as a product, these including catalytic activity and inhibition studies of react-CT, the reaction of react-CT with PMSF and subsequent behavior, quantitative $^{14}$C tracer studies on the reaction of labeled PMS-CT with $H_2O_2$ (monitoring $^{14}$C loss and active site regain simultaneously), and finally, chemical analysis. Reactivation with $CH_3OOH$ gave similar results and supported the conclusion above.

Various data show that the effect of peroxide is not to labilize sulfonate by attacking another moiety of the protein. From Table IV, it is apparent that met-192 sulfoxide-chymotrypsin [CT($H_2O_2$)] is quantitatively sulfonated on reaction with PMSF, and the resultant PMS-CT($H_2O_2$) is stable in buffer alone. Accompanying graphs and Table V show that reactivation occurs when this material is treated with $H_2O_2$, and this reaction is almost identical in rate to the native PMS-CT reaction with $H_2O_2$. A survey of potential nucleophiles uncovered no others that caused loss of $^{14}$C from labeled sulfonyl enzyme or appearance of catalytic activity with anything approaching the efficacy of $H_2O_2$.

A number of studies have been done on the interaction of chymotrypsin with $H_2O_2$.[19,24,29,30] At pH 3, met-192 is oxidized to the sulfoxide and much more slowly, or in 7.6 *M* urea, so is met-180.[24] $H_2O_2$ in approximately neutral buffer was reported by one group to be unreactive toward the enzyme as far as activity is concerned, but with 10% dioxane at pH 8 tryptophans begin to be destroyed, and activity is lost.[29] Schachter, *et al.*, reported that at pH 6.7 met-192 is preferentially oxidized.[30] When only met-192 is oxidized as the sulfoxide the enzyme has only somewhat altered catalytic properties,[19,24,25] but the disulfoxide material has very poor catalytic activity, and tryptophan destruction results in complete loss of activity.[24,29] These prior results and our data are in agreement and show that $H_2O_2$, as a side reaction, oxidizes just met-192 during incubation.

The reactivation reaction between PMS-CT and $H_2O_2$ has some special kinetic properties. First, the rate is considerably faster at the pH's used than is the model system. For example, at pH 7 the observed first-order rate constant for *n*-butyl methanesulfonate aqueous hydrolysis[4] is $2.1 \times 10^{-6}$ sec$^{-1}$, while PMS-CT is not observably reactive (at least 100-fold less than this). The addition of 0.6 *M* $H_2O_2$ has no effect on the organic system until pH 10, while the enzymic system at pH 7 shows loss of $^{14}$C (and corrected activity regain) with a $k_{obsd}$ of $9.8 \times 10^{-6}$ sec$^{-1}$. The second-order rate constant for the organic sulfonate and $HO_2^-$ is $96 \times 10^{-5}$ $M^{-1}$ sec$^{-1}$, at pH 7, this giving a calculated pseudo-first-order rate constant for 0.6 *M* $H_2O_2$ of about $3 \times 10^{-8}$ sec$^{-1}$. The enzyme system is thus providing a highly efficient route for the reaction.

Secondly, the pH dependence of reactivation shows base dependence with a leveling off at around pH 8 and an inflection point at around 7. (This is also true for various nucleophiles with phosphorylated and carbamylated chymotrypsin.[31]) This is the approximate $pK_a$ seen in chymotrypsin-catalyzed acylation and deacylation reactions with normal substrates and seems to indicate involvement of the charge relay system (or imidazole) acting in a base catalysis sense. (Base catalysis is not very surprising, since $HO_2^-$ is a far better nucleophile than is $H_2O_2$.) Finally, the reactivation is specific for $H_2O_2$, with greatly decreased efficacy (but the same general behavior) of $CH_3OOH$, and has no effect with a variety of other powerful nucleophiles. (It might be worthwhile to point out that, in respect to their $pK_a$'s, amine and oxygen nucleophiles have similar reactivity toward sp$^3$ C (*i.e.*, similar Brønsted $\beta$'s) but that oxygen nucleophiles are generally much more reactive than nitrogen ones toward the harder carbonyl carbon and, in the one case studied,[32] sulfonyl sulfur.[33,34]) The active site of chymotrypsin is specifically organized to provide highly efficient acyl–oxygen scission, much more akin geometrically and electronically to sulfonyl–oxygen than alkyl–oxygen cleavage.

The choice of pathways, then, breaks down to those depicted below.

$$\text{E-CH}_2\text{OSO}_2\text{R} + \text{H}_2\text{O}_2 \longrightarrow \text{E-CH}_2\text{OH} + \text{RSO}_2\text{OO}^- \xrightarrow{\text{rapid}} \text{RSO}_2\text{O}^- \quad (3)$$

$$\text{E-CH}_2\text{OSO}_2\text{R} + \text{H}_2\text{O}_2 \longrightarrow \text{E-CH}_2\text{OOH} + \text{RSO}_2\text{O}^- \xrightarrow{\text{rapid}} \text{E-CH}_2\text{OH} \quad (4)$$

(29) Y. Hachimori, H. Horinishi, K. Kurihara, and K. Shibata, *Biochim. Biophys. Acta*, **93**, 346 (1964).

(30) H. Schachter, K. A. Halliday, and G. H. Dixon, *J. Biol. Chem.*, **238**, 3134 (1963).

(31) B. F. Erlanger, A. W. Cooper, and W. Cohen, *Biochemistry*, **5**, 190 (1966); W. Cohen and B. F. Erlanger, *J. Amer. Chem. Soc.*, **82**, 3928 (1960).

(32) J. L. Kice and E. Legan, *ibid.*, **95**, 3912 (1973).

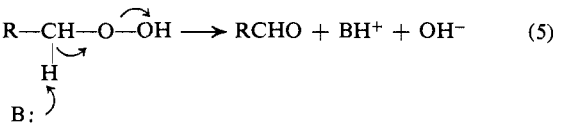
(33) M. L. Bender, "Mechanisms of Homogeneous Catalysis from Protons to Proteins," Wiley-Interscience, New York, N. Y., 1971, p 148 ff.

(34) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, Chapters 2 and 10.

In the preceding paper,[4] it is shown that *n*-butyl methanesulfonate is not attacked by $H_2O_2$ but readily undergoes SN2 displacement at carbon with $HO_2^-$. Neopentyl methanesulfonate was not attacked over long time periods (thus attack at sulfonyl sulfur was quite slow). On this basis eq 4 seems attractive. However, eq 3 is also quite plausible.

Kice has very recently shown that hydroperoxide ion will attack sulfonyl sulfur (in an $\alpha$-disulfone) to cause hydrolysis,[32] and several other cases of S–O scission of sulfonate esters are known for unreactive alcohol portions (phenyl and neopentyl esters) and also for intramolecular attack.[35] As just pointed out, this route is also one for which the enzyme is likely to be somewhat catalytically helpful. Of course, the contrary evidence is isolation of normal sulfonate and noninterception of persulfonate, but it is conceivable that persulfonate in this case breaks down very rapidly (with $H_2O_2$, for example, to form sulfonate, $O_2$, and $H_2O$).

The alkyl–oxygen route, involving formation of enzyme hydroperoxide, is of course the one that is predicted from the organic system. This process is akin to one that Koshland observed in generating "anhydrochymotrypsin" *via* elimination from tosyl-CT.[36] The displacement of sulfonate on sulfonyl subtilisin with thiolacetate and subsequent deacylation to yield thiol-subtilisin is one example of a substitution reaction taking place at the active site of an extremely similar (as regards active site) enzyme.[37,38] In organic systems, primary alkyl hydroperoxides are quite stable under the reaction conditions that obtain here, so that we had earlier deemed unlikely a breakdown of any possible serine-195 hydroperoxide functionality. Perhaps, however, there is at least one reason to expect such a moiety in this case to be relatively labile. We can certainly rule out both homolytic decomposition and the normal mode or primary hydroperoxide ionic decomposition (but under much more rigorous conditions than here) *via* elimination of the elements of water by basic attack (eq 5) at an $\alpha$-CH bond. This is really quite



(5)

unlikely (also we obtain serine-OH). Nucleophilic attack at peroxide oxygen, however, is a well-documented and rather general reaction (eq 6).[39] The better $XO^-$

$$XOOY + N^- \longrightarrow XO^- + YON \qquad (6)$$

is as a leaving group the more facile the reaction. These reactions, like those of disulfides, may also be acid catalyzed, since then the leaving moiety is XOH. In the system under consideration here, XOOY is $CT\text{–}CH_2OOH$ (or $CT\text{–}CH_2OOCH_3$) and $N^-$ could reasonably be $OH^-$. The products would be $CT\text{–}CH_2OH$ and $H_2O_2$ (or $CH_3OOH$). This process might be unusually facile for $CT\text{–}CH_2OOH$ ($CT\text{–}CH_2OOCH_3$) since the active site is designed to provide hydrogen bonding to the serine alcoholic oxygen (or stabilize an alkoxide type form), exactly the kind of process that would facilitate nucleophilic attack at the ($\beta$) peroxide oxygen.[40]

Finally, we may consider the nature of the $H_2O_2$ specific interaction with sulfonyl enzyme. The pH dependence and the relative reactivities of $H_2O_2$ and $HO_2^-$ make it almost inescapable to conclude that the enzyme is acting as a base catalyst in this desulfonation reaction. The acidic proton of $H_2O_2$ could be removed by the imidazole while in the correct orientation for oxygen attack at either $CH_2$ or S of the sulfonate ester.

**Acknowledgments.** We wish to thank Dr. Eugene Zeffren for suggesting the Polar Brill Blue experiment and providing some insight into persulfonic acid chemistry.

(35) See discussion in ref 4.

(36) H. Weiner, W. N. White, D. G. Hoare, and D. E. Koshland, Jr., *J. Amer. Chem. Soc.*, **88**, 3851 (1966).

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(40) The question of initial C–O *vs.* S–O cleavage is in principle answerable by using $H_2{}^{18}O_2$ to reactivate PMS-CT followed by analysis of the $^{18}O$ content of the resulting phenyl methanesulfonate. For various experimental reasons, we have not performed such an experiment up to this time.