The Reactions of Hydrogen Peroxide and Some of Its Derivatives with Uracil, Thymine, and Thymidine 5′-Phosphate

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Hydrogen peroxide undergoes both polar and free-radical reactions with uracil and thymine derivatives. Near neutrality, free-radical pathways are the most important for the reaction with thymidine 5′-phosphate. At more alkaline pH values, the predominant reaction is one in which the anion of the hydroperoxide attacks the neutral species of the substrate. The pH-rate profiles of these reactions show maxima midway between the pK_a values of the two reacting species. In addition to hydrogen peroxide, methyl hydroperoxide, *tert*-butyl hydroperoxide, peroxyacetic acid, and *m*-chloroperoxybenzoic acid show the same type of behavior. Thymine is less reactive than uracil in all cases.

Selective chemical modification of nucleic acids is an area of increasing interest for the study of the structure and function of these polymers. Rational application of these modifications to the polymers requires detailed knowledge of the mechanisms of the reactions with the monomeric units. We have reported a study of the reaction of *m*-chloroperoxybenzoic acid with nucleic acid components.[1,2] Uracil, thymine, and their nucleosides and nucleotides were each shown to undergo a reaction which exhibited a rate maximum in the alkaline range. There was no evidence for free-radical involvement. The results were accounted for by a mechanism in which the neutral substrate was attacked by the anion of the peroxy acid to form products which led to ring cleavage. On the other hand, the effects of hydrogen peroxide on nucleic acids and their components have generally been regarded as the results of radical-forming processes.[3,4] Since the results of Priess and Zillig[5] showed a strong pH dependence for the rate of reaction of hydrogen peroxide with both uracil and thymine, we thought it probable that a part of the reactivity of hydrogen peroxide with these nucleic acid components might be due to attack by the hydroperoxide anion rather than the hydroxyl radical. We have, therefore, reinvestigated the reaction of hydrogen peroxide and several of its derivatives with some nucleic acid components. We also report some comparative data on the reactivity of hydrazine and hydroxylamine.[4,6,7]

Materials and Methods.—Reactions of the peroxides in the alkaline region were followed by iodometric measurement of the concentration of peroxide as a function of time using initially equal concentrations of both reactants (*ca.* 3×10^{-3} *M*). Iodine was liberated quantitatively from the reaction of methyl hydroperoxide and of *tert*-butyl hydroperoxide with iodide in dilute acetic acid by allowing reaction times of 1 hr and 1.5 hr, respectively, and in the case of hydrogen peroxide, by the use of a molybdate catalyst.[8] The reactions of the peroxy acids with iodide were fast. Apparent second-order rate constants, calculated for total substrate and peroxide (*i.e.*, no correction for the per cent ionized), were obtained from slopes of $x/a(a-x)$ *vs.* time plots. The error in the rate constants is of the order of ±4%. The necessary blank corrections were made.[2] EDTA (1×10^{-4} *M*) was added in all experiments (except some of those with thymidine 5′-phosphate) to minimize metal-catalyzed chain decomposition.

The reactions of thymidine 5′-phosphate with hydrogen peroxide were followed by the method of Rhaese, *et al.*[9]

The reactions of hydroxylamine (salt-free) and hydrazine with uracil were followed by measurement of the decrease in the absorption of uracil at 258.5 mμ following dilution of aliquots 100-fold in pH 7 buffer. Reactions were run under pseudo-first-order conditions (*ca.* 1×10^{-2} *M* uracil and 0.5 to 1.2 *M* reagent). Pseudo-first-order rate constants were evaluated from log absorbancy *vs.* time plots, and the data restricted to 5–10% conversion in order to avoid interference from product absorption. The reagents were standardized according to Vogel.[10]

Phosphate and carbonate buffers were used throughout. Reagents were from commercial sources except for methyl hydroperoxide which was prepared by the procedure of Rieche and Hitz[11] as modified by Behrman, *et al.*[12]

Ultraviolet spectra were recorded on a Perkin-Elmer Model 202 instrument; extinction coefficients were measured using a Hitachi Perkin-Elmer Model 139. Urea, oxaluric acid, pyrazolone-3, and isoxazolone-5 were identified according to published procedures.[2,6,13] Urea was estimated quantitatively by the method of Coulombe and Favreau.[14]

Results

Products.—The products from the reactions of uracil and all of the peroxides in the alkaline region consisted of ring-cleavage fragments. All gave urea as one of the products. This was identified by paper chromatography on Whatman No. 1 paper using 1-butanol–

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TABLE I

Nucleophile[a]	pK_a'	k_{max}, M^{-1} min^{-1}	T, °C	Obsd pH_{max}	Calcd pH_{max}[b]	E_a, kcal mol^{-1}	Relative rate at 60°, pH 9	U/T rate[c] ratio
m-Chloroperoxybenzoic acid	7.4[d]	8.3	40	8.7–8.9	8.3	14.5	1576	4.4
Peroxyacetic acid	8.0[e]	3.3	40	8.6–9.0	8.6	14.5	688	4.1
Hydrogen peroxide	10.8[f]	0.74	60	9.8–9.9	9.8	15	24	4.1
Methyl hydroperoxide	10.6[g]	0.32	60	9.5–9.7	9.7	14.5	13	
tert-Butyl hydroperoxide	12.0[f]	0.04	60	9.8–10.3	10.4	20	1	2.2
Hydroxylamine	5.7[h]	0.067	40	7.3–7.6	7.4	10	4.3	
Hydrazine	7.1[h]	0.029	50	9.1–9.6	8.1	13	2.6	

[a] The ionic strength varied from 0.1 to 0.3 M for all cases except for *tert*-butyl hydroperoxide for which the range was 0.3–0.5 M. [b] The average of the pK_a' values for the indicated nucleophile and for uracil. [c] The ratio of the second-order rate constants for the reactions of uracil and thymine with the indicated peroxide at the pH of the observed rate maximum for uracil. [d] J. F. Goodman, P. Robson, and E. R. Wilson, *Trans. Faraday Soc.*, **58**, 1846 (1962). [e] E. Koubek, M. L. Haggett, C. J. Battaglia, K. M. Ibne-Rasa, H. Y. Pyun, and J. O. Edwards, *J. Amer. Chem. Soc.*, **85**, 2263 (1963). [f] W. F. Sager and J. C. Hoffsommer, *J. Phys. Chem.*, **73**, 4155 (1969). [g] J. E. McIsaac, Jr., H. A. Mulhausen, and E. J. Behrman, Abstracts, 156th National Meeting of the American Chemical Society, Sept 1968, ORGN 70. [h] R. M. Izatt and J. J. Christensen in "Handbook of Biochemistry," H. A. Sober, Ed., Chemical Rubber Publishing Co., Cleveland, Ohio, 1968, pp J49 ff. This reference also gives pK_a' values for uracil, 9.1 (40°), 8.8 (60°).

ethanol–water (4:1:5, v/v) and 1-butanol–acetic acid–water (2:1:1, v/v) as solvents. Ehrlich's reagent was used to detect the spots.[2] Elution and rechromatography gave R_f values of 0.36 and 0.77, respectively. Prior treatment with urease eliminated these spots. The quantitative determination of urea is described later. Oxaluric acid was identified on similar chromatograms from the reactions of uracil with *m*-chloroperoxybenzoic acid, peroxyacetic acid, and hydrogen peroxide by coincidence of its R_f value with authentic material, by reaction with Ehrlich's reagent, and by hydrolysis in dilute HCl to oxalic acid and urea.[2] Oxaluric acid and the other expected intermediates in the hydrolytic decomposition of the initial addition product[2] could not be found for the more slowly reacting peroxides, presumably because their rates of decomposition exceed their rates of formation. The products of the reactions of thymine with *m*-chloroperoxybenzoic acid and with hydrogen peroxide are hydroxyacetone and urea.[2,15]

The hydrazine–uracil reaction mixture upon paper chromatography using 1-butanol–0.6 N ammonia (6:1) gave two spots corresponding to urea and pyrazolone-3.[6] Examination of the hydroxylamine–uracil reaction mixture after treatment with 1 N NaOH[13] showed the presence of urea and isoxazolone-5.

Kinetics.—Table I and Figure 1 present our kinetic results with uracil. The figure shows that in each case the pH-rate profile is a bell-shaped curve. The table reports the maximum observed second-order rate constant, the temperature and pH at which it was observed, as well as the activation energy for the reaction. Table I also presents data for the reaction of thymine with four peroxides at those pH values for which the corresponding reaction with uracil exhibits a rate maximum. Typical second-order plots are shown in Figure 2.

Effects of Radical Traps.—Table II shows that neither allyl alcohol nor acrylamide has any substantial effect on the extent of urea formation from uracil or from thymine at pH 9.8. Likewise, in experiments in which thymidine 5′-phosphate was monitored at 260 mμ, there was no difference in the rate of change in absorbancy with and without allyl alcohol under the following conditions: (1) 37°, pH 9.8, 1.5×10^{-2} M hydrogen peroxide, 1.0×10^{-2} M thymidine 5′-phosphate, $\pm 1 \times 10^{-2}$ M allyl alcohol; (2) 50°, pH 10.3, 2.5×10^{-2} M hydrogen peroxide, 1.5×10^{-2} M thymidine 5′-phosphate, $\pm 1.5 \times 10^{-2}$ M allyl alcohol. Figure 3 shows, in contrast, that at pH 7.4 the reaction of thymidine 5′-phosphate with hydrogen peroxide in the presence of ferric ions is markedly inhibited by the presence of allyl alcohol. In other experiments with thymidine 5′-phosphate at pH 7.4, we have shown that in the absence of allyl alcohol the omission of ferric ions or the addition of

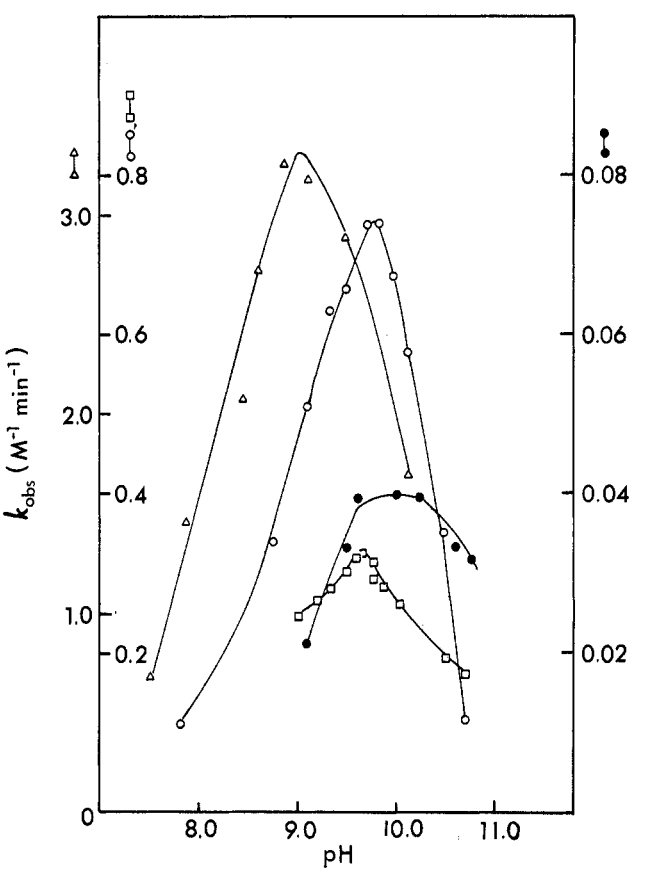

Figure 1.—pH-rate profiles for the reactions of uracil with hydrogen peroxide (○—○), methyl hydroperoxide (□—□), and *tert*-butyl hydroperoxide (●—●) at 60°, and for peroxyacetic acid (△—△) at 40°. Analogous data for the reaction with *m*-chloroperoxybenzoic acid are given in ref 2.

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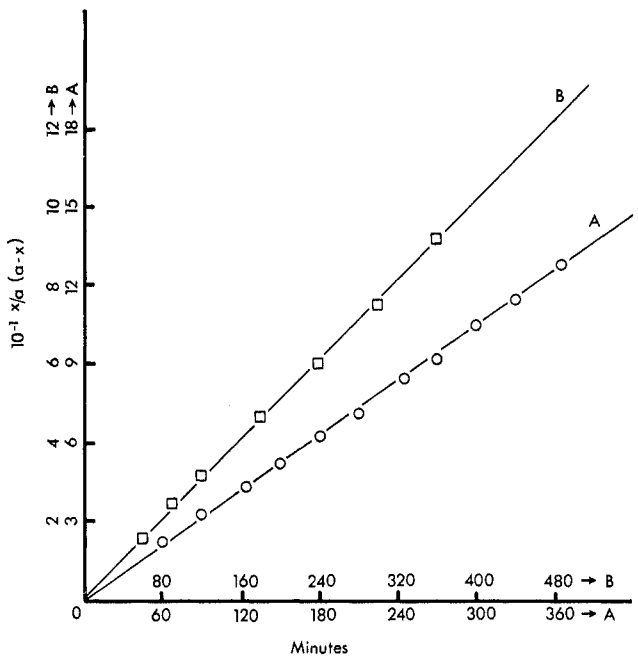

Figure 2.—Second-order plots for the reactions of uracil and thymine with H_2O_2 in the alkaline region. A, hydrogen peroxide and uracil concentrtions were 3.025×10^{-3} M initially, 50°, pH 9.95 in 0.05 M carbonate buffer. B, hydrogen peroxide and thymine concentrations were 5.665×10^{-3} M initially, 60°, pH 9.8 in 0.05 M carbonate buffer; a is the initial concentration of hydrogen peroxide and x is the concentration at time t.

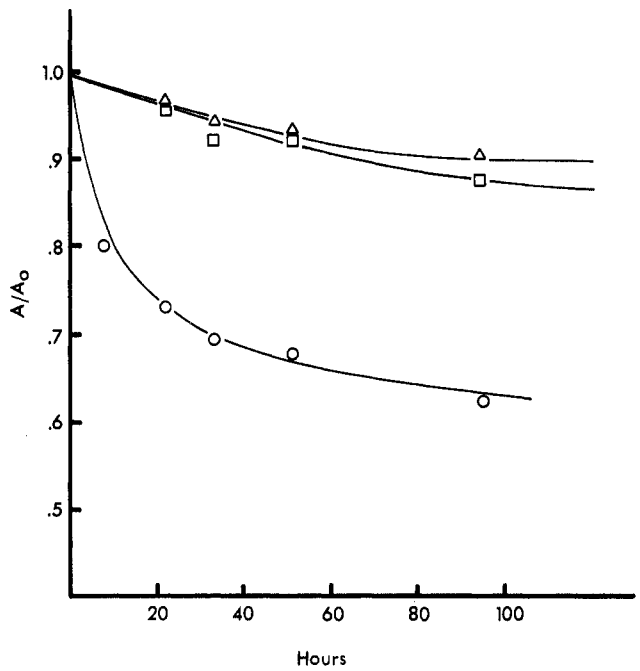

Figure 3.—The reaction of thymidine 5′-phosphate with H_2O_2 at pH 7.4. All reaction mixtures contained 5.6×10^{-2} M hydrogen peroxide, 8.6×10^{-3} M thymidine 5′-phosphate, and 1×10^{-1} M phosphate buffer, pH 7.4. O—O also contained 1×10^{-5} M ferric chloride; □—□, 1×10^{-5} M ferric chloride and 5×10^{-2} M allyl alcohol; △—△, 1×10^{-4} M EDTA and 5×10^{-2} M allyl alcohol. After 22 hr, hydrogen peroxide concentrations were 3.5×10^{-2} M, 3.8×10^{-2} M, and 3.3×10^{-2} M, respectively. After 94 hr, the corresponding values were 2.5×10^{-2} M, 1.8×10^{-2} M, and 1.6×10^{-2} M. Absorbancy measurements were made following dilution of aliquots 100-fold in the same buffer.

TABLE II

UREA FORMATION FROM THE REACTION OF HYDROGEN PEROXIDE WITH URACIL AND THYMINE

1. Uracil, 0.02 M; H_2O_2, 0.04 M, pH 9.8, 60°

	Mol of urea/mol of uracil	
Allyl alcohol, M	5 hr	22 hr
0	0.65	0.95
0.01	0.70	0.97
0.02	0.63	0.93

2. Uracil, 0.04 M; H_2O_2, 0.02 M, pH 9.8, 60°

	Mol of urea/mol of H_2O_2	
Acrylamide, M	5 hr	22 hr
0	0.40	0.48
0.01	0.48	0.45
0.02	0.35	0.38

3. Thymine, 0.02 M; H_2O_2, 0.06 M, pH 9.8, 60°

	Mol of urea/mol of thymine	
Allyl alcohol, M	5 hr	22 hr
0	0.23	0.87
0.01	0.22	0.82
0.02	0.23	0.85

EDTA without ferric ions has little effect either on the rate of loss of absorbancy or on the rate of loss of hydrogen peroxide.

Discussion

Our evidence suggests that the rate maxima which we observe in the alkaline range are the result of bimolecular reactions between the peroxyanion and the neutral substrate. This mechanism requires first-order dependence on each of the reactants and an observed rate maximum midway between the pK_a values of the two reacting species. The rate falls off on the alkaline side because of formation of the anion of the substrate; the decrease on the acid side is due to protonation of the nucleophile. An alternative reaction between the un-ionized peroxide and the ionized base seems unlikely because in each case thymine reacts more slowly than uracil. Nucleophilic attack by the peroxyanion is also consistent with a large body of evidence in other systems.[16,17] We consider, because of the similarity of products, that the same reaction sequence postulated for the case of *m*-chloroperoxybenzoic acid[2] occurs for all of the hydroperoxides (Scheme I). For the case of hydrogen peroxide, it could be argued that the position of the rate maximum is also consistent with the idea that the reactive species is the anion of the hydroxyl radical (pK_a 11.9 ± 0.2[18]). There are several considerations which weigh against the participation of the hydroxyl radical, however. (1) Neither allyl alcohol nor acrylamide affects the rate or extent of formation of urea. Were the reaction sequence to involve a significant free-radical contribution, the addition of a radical trap known to react with hydroxyl radicals such as allyl alcohol or acrylamide[19,20] would result in a decrease in both the yield and rate of formation of a product of the sequence. (2) Homolysis of the alkyl substituted hydroperoxides would produce an alkoxide radical and a hydroxyl radical. The reactions of the alkoxide radical would not be pH-dependent. Were the reactivity of the substituted peroxides due to the reaction of the hydroxyl radical, the

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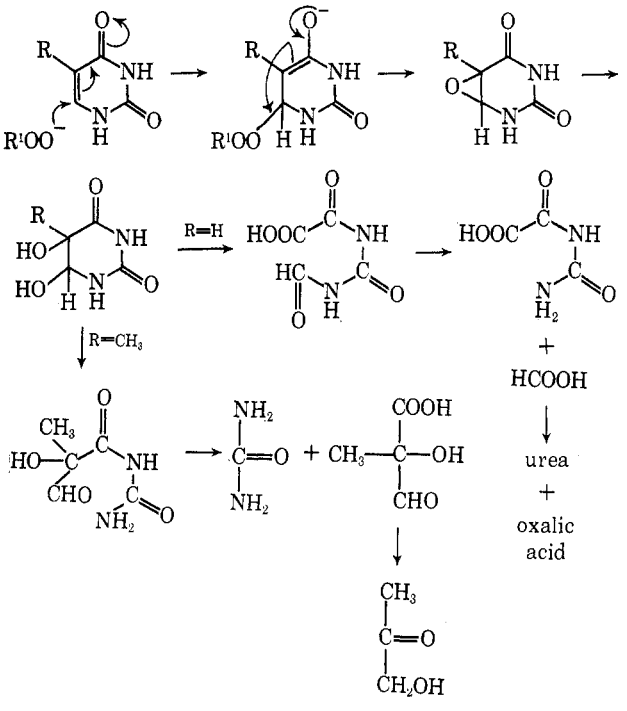
SCHEME I



rate maximum for a given substrate would be at the same pH for all of the hydroperoxides. (3) The observation of second-order kinetics, although conceivable for a radical pathway, is much commoner for nonradical pathways. For example, a nonchain radical mechanism involving rate-limiting homolysis of the peroxide and subsequent attack by the hydroxyl radical on the substrate would show no kinetic dependence on substrate concentration. In contrast to the evidence suggesting the predominance of polar reactions at alkaline pH values, the demonstration that allyl alcohol decreases the rate of loss of thymidine 5′-phosphate in the presence of hydrogen peroxide at pH 7.4 approximately sixfold is positive evidence that a radical pathway is involved under these conditions. There is some reaction of hydrogen peroxide with allyl alcohol, but the change in concentration of hydrogen peroxide due to this reaction is negligible (less than 10% at the highest concentration of allyl alcohol used) up to 30 hr and hence can only account for a small portion of the effect which we observe. The kinetics of the disappearance of thymidine 5′-phosphate under these conditions are complex since Rhaese, *et al.*,[9] have shown that several different reactions, all of which lead to decreases in the absorbancy, occur simultaneously.

Registry No.—Hydrogen peroxide, 7722-84-1; uracil, 66-22-8; thymine, 65-71-4; thymidine 5′-phosphate, 365-07-1; methyl hydroperoxide, 3031-73-0; *tert*-butyl hydroperoxide, 75-91-2; peroxyacetic acid, 79-21-0.

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Chemistry of Cephalosporin Antibiotics. XXI. Conversion of Penicillins to Cephalexin[1]

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A laboratory synthesis from the biosynthetic penicillins is described for cephalexin (**7**), an orally active deacetoxycephalosporin antibiotic. Penicillins V and G were converted to sulfoxide trichloroethyl esters **3a** and **3b**, respectively, by an esterification to compounds **2a** and **2b** followed by sulfoxidation. The sulfoxide esters **3a** and **3b** were rearranged thermally to their corresponding deacetoxycephalosporin esters **4a** and **4b**. Proof of structure for **4a** and **4b** was supplied by their independent syntheses from 7-aminodeacetoxycephalosporanic acid (**9**). N-Deacylation of **4a** and **4b** afforded a common amino ester, 7-aminodeacetoxycephalosporanic acid trichloroethyl ester (**5d**). Compound **5d** was reacylated in mixed anhydride coupling reactions with *N*-trichloroethyloxycarbonyl-D-α-phenylglycine and with *N*-*tert*-butoxycarbonyl-D-α-phenylglycine. The doubly protected cephalexin derivatives **6** and **12** were deblocked yielding cephalexin in good yield.

Previous publications from these laboratories have disclosed the *in vitro* and *in vivo* biological,[2] toxicological,[3] and pharmacological[3,4] properties of the orally absorbed deacetoxycephalosporin antibiotic, cephalexin (**7**).

We have examined several synthetic routes to cephalexin. One already described by Ryan, *et al.*,[5] proceeds from cephalosporin C through 7-aminodeacetoxycephalosporanic acid (7-ADCA, **9**). Another, which forms the basis of this report, stems from the work of Morin, *et al.*,[6] on the conversion of penicillin sulfoxides to deacetoxycephalosporins. The latter demonstrated that phenoxymethylpenicillin sulfoxide methyl ester, when heated under reflux in toluene with *p*-toluenesulfonic acid, rearranged in about 20% yield to the corresponding deacetoxycephalosporin methyl ester. A plausible mechanism offered was a cleavage of the S–C bond in the thiazolidine ring of the penicillin sul-

(1) Cephalexin is the generic name for 7-(D-2-amino-2-phenylacetamido)-3-methyl-3-cephem-4-carboxylic acid; cephalexin monohydrate; KEFLEX, Lilly.

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