Guschlbauer, W. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 1441.
Hartman, K. A., and Rich, A. (1965), *J. Am. Chem. Soc. 87*, 2033.
Holcomb, D. N., and Timasheff, S. N. (1968), *Biopolymers 6*, 513.
Inman, R. B. (1964a), *J. Mol. Biol. 9*, 624.
Inman, R. B. (1964b), *J. Mol. Biol. 10*, 137.
Inman, R. B., and Baldwin, R. L. (1964), *J. Mol. Biol. 8*, 452.
Kato, K., Goncalves, J. M., Houts, G. E., and Bollum, F. J. (1967), *J. Biol. Chem. 242*, 2780.
Kulikowski, T., Zmudzka, B., and Shugar, D. (1969), *Acta Biochim. Polon. 16*, No. 2 (in press).
Lee-Huang, S., and Cavalieri, L. F. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 1116.
Lee-Huang, S., and Cavalieri, L. F. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 1022.
Lewin, S., and Humphreys, D. A. (1966), *J. Chem. Soc. B*, 210.
Martin, D. M. G., and Reese, C. B. (1967), *Chem. Commun.*, 1275.
Michelson, A. M., Massoulie, J., and Guschlbauer, W. (1967), *in* Progress in Nucleic Acid Research and Molecular Biology, Davidson, J. N., and Cohn, W. E., Ed., New York, N. Y., Academic, p 83.
Rabczenko, A., and Szer, W. (1967), *Acta Biochim. Polon. 14*, 369.
Radding, C. M., Josse, J., and Kornberg, A. (1962), *J. Biol. Chem. 237*, 2869.
Riley, M., Maling, B., and Chamberlin, M. J. (1966), *J. Mol. Biol. 20*, 359.
Shugar, D., and Szer, W. (1962), *J. Mol. Biol. 5*, 580.
Stevens, C., and Felsenfeld, G. (1964), *Biopolymers 2*, 293.
Szer, W., and Shugar, D. (1966), *J. Mol. Biol. 17*, 174.
Szer, W., Swierkowski, M., and Shugar, D. (1963), *Acta Biochim. Polon. 10*, 87.
Ts'o, P. O. P., Rapaport, S. A., and Bollum, F. J. (1966), *Biochemistry 5*, 4153.
Wenkstern, T. B. (1966), *Dokl. Akad. Nauk SSSR 170*, 718.
Yoneda, M., and Bollum, F. J. (1965), *J. Biol. Chem. 240*, 3385.
Zmudzka, B., Bollum, F. J., and Shugar, D. (1968), Abstracts, Vth Annual Meeting, Federation of European Biological Societies, Prague, Vol. 1, p 70.

The Reaction of Nucleic Acid Components with *m*-Chloroperoxybenzoic Acid*

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ABSTRACT: We have studied the kinetics of the reactions of the commonly occurring nucleic acid bases, nucleosides, and nucleotides with *m*-chloroperoxybenzoic acid as a function of pH. The pH–rate profiles are bell-shaped curves with maxima which fall into one of two groups: cytosine, adenine, and their derivatives show maxima on the acid side of the pK_a of the peroxy acid, while uracil, thymine, guanosine, and their derivatives show maxima on the alkaline side. The products from the first group are *N*-oxides, while the second group gives ring-cleavage products. Substituent effects allow us to characterize the predominant mechanism for *N*-oxide formation from cytosine as nucleophilic attack of the peroxy acid anion on the cationic substrate, while *N*-oxide formation from adenine occurs *via* electrophilic attack of the un-ionized peroxy acid on the neutral substrate. The initial attack leading to ring cleavage of the uracil group occurs *via* nucleophilic attack of the peroxy acid anion at the 5,6-double bond of the neutral substrate.

The reactions of peroxides with nucleic acids and their components have been studied previously in a number of laboratories. Peroxycarboxylic acids have been used to convert cytosine, adenine, guanine, and some of their derivatives into *N*-oxides (Cramer and Seidel, 1963; Delia *et al.*, 1965; Seidel, 1967; Cramer *et al.*, 1963; Delia and Brown, 1966); hydrogen peroxide in acetic acid has also been used for the synthesis of adenine 1-*N*-oxide and some derivatives (Stevens *et al.*, 1958, 1959; McCormick, 1966; Sigel and Brintzinger, 1965). In addition to *N*-oxide formation, however, a number of other reactions of peroxides with nucleic acid components have been observed, and these include addition of hydrogen peroxide to the 5,6-double bond of the pyrimidines (see Rhaese *et al.*, 1968), pyrimidine ring cleavage in alkaline solutions of hydrogen peroxide (Priess and Zillig, 1965), and both cleavage of the *N*-glycosidic bond and breakage of the sugar–phosphate backbone (Rhaese and Freese, 1968).

Peroxides are capable of reacting in a number of fundamentally different ways (Behrman and Edwards, 1967). They may undergo polar reactions in which the peroxide serves either as nucleophile or electrophile, or, as is usually assumed, but fre-

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quently not the case, the peroxide may first undergo homolysis to radicals. It was because of these possibilities that we undertook a systematic study of the reactions of one peroxide with nucleic acid components anticipating that, as the differently ionized forms of both the peroxide component and the heterocyclic base become predominant, interesting differences in reactivity would ensue. We are also concerned with the lack of systematic quantitative data on the rates of reaction of the heterocyclic bases of the nucleic acids with nucleophilic, electrophilic, and free-radical reagents. Differences in reactivity do exist, but their potential in the areas of controlled alteration of nucleic acid structure has not been fully exploited because it is not sufficiently known. A preliminary report on some aspects of these studies has appeared (Subbaraman *et al.*, 1968).

Experimental Procedures

Kinetics. Reactions were routinely run under second-order conditions, using equimolar concentrations of both the peroxy acid and the substrate, generally of the order of 3×10^{-3} M. The course of the reactions was followed by measurement of the concentration of the peroxy acid as a function of time using standard iodometric techniques (Schwartz and Blumbergs, 1964). Plots of $x/a(a - x)$ against time were satisfactorily linear and the apparent second-order rate constants were obtained from the slopes of these plots. We estimate the error in the rate constants to be of the order of $\pm 3\%$. The second-order dependence was confirmed for three typical substrates (cytidine, uridine, and pyridine) by running a series of reactions for each case under pseudo-first-order conditions. Plots of the pseudo-first-order constants against substrate concentrations were linear and gave rate constants in agreement with the values obtained from second-order plots.

The kinetic analysis is complicated by the fact that the peroxy acid is undergoing two parallel reactions: the autodecomposition of the peroxy acid itself which is second-order in peroxy acid (Behrman and Edwards, 1967) and the reaction of the peroxy acid with the substrate which we may initially assume to be first-order in each component. This general kinetic situation has been discussed by Russell (1961). The expected rate law is of the form $-d[P]/dt = k_1[P]^2 + k_2[P][S]$, where P is the peroxy acid and S is the substrate. We have treated this situation experimentally with the following set of approximations. Since we have set the initial concentrations of P and S equal, $[P]^2 = [P][S]$ and $-d[P]/dt = (k_1 + k_2)[P][S]$ under initial conditions. The observed rate constant, k_{obsd}, is then equal to $k_1 + k_2$. k_1, the second-order rate constant for the autodecomposition of the peroxy acid was measured independently and an approximate value of k_2 obtained by subtraction of k_1 from k_{obsd}. k_1^{max} was found to have a value of 2.9 M^{-1} min^{-1} at pH 7.7, 40°. Curci and Modena (1965) have handled this kinetic situation using different assumptions and conditions.

In order to minimize metal-catalyzed chain decomposition of the peroxy acid (Ball and Edwards, 1956, 1958), 1×10^{-4} M EDTA was added to all runs. Sodium or potassium acetate, phosphate, and carbonate buffers were used and showed no specific effects other than those expected from an alteration in the ionic strength. The rate of disappearance of peroxy acid was always faster in the presence of borate buffers and this we attribute to the formation of peroxyboric acid (Ross and Edwards, 1967).

Materials and Methods

8-Bromoadenosine, *m*-chloroperoxybenzoic acid, and the substituted pyridines, were purchased from the Aldrich Chemical Co., and 5-bromocytosine from the Cyclo Chemical Corp. All other nucleic acid components were obtained from The Sigma Chemical Co. or from the Mann Research Laboratories in satisfactory states of purity for these studies as checked by their ultraviolet spectra (Beaven *et al.*, 1955). 6-Methylcytosine monohydrate was synthesized by the following procedure rather than by the original route of Johns (1908): β-aminocrotononitrile (Aldrich) was condensed with thiourea following the directions of Polonovski (Polonovski *et al.*, 1948) to yield 4-amino-6-methyl-2-pyrimidinethione. This material was converted in 75% yield into the 2-oxo compound (6-methylcytosine) by alkylation with chloroacetic acid and acid-catalyzed hydrolysis according to the procedure described by Brown for thiocytosine (Brown, 1950; see also Arantz and Brown, 1968). Cytosine 3-*N*-oxide was synthesized by reaction of equimolar amounts of cytosine hemihydrate and *m*-chloroperoxybenzoic acid at about pH 5 in the presence of EDTA. The product corresponds in all its properties to cytosine 3-*N*-oxide (4-amino-2-oxo-pyrimidine 3-*N*-oxide) prepared by an unequivocal ring closure synthesis (Klötzer, 1965) as well as to the material prepared by the oxidation of cytosine with *m*-chloroperoxybenzoic acid in glacial acetic acid (Delia *et al.*, 1965) and to the product prepared by the oxidation of cytosine at pH 7 with monoperoxyphthalic acid (Cramer and Seidel, 1963). Oxaluric acid was purchased from the Nutritional Biochemical Corp. Thymine glycol was synthesized by the procedure of Baudisch and Davidson (1925).

Ultraviolet spectra were recorded on a Perkin-Elmer Model 202 and infrared spectra on a Perkin-Elmer Model 237B. Ionization constants were determined potentiometrically following the procedures of Albert and Serjeant (1962).

Urea was identified on chromatograms by its R_F value in several solvent systems and by reaction with Ehrlich's reagent (Fink *et al.*, 1956); its presence was confirmed by showing the disappearance of this spot following treatment of aliquots with urease. Formic acid was detected following reduction to formaldehyde by reaction with chromotropic acid (Feigl, 1966). Oxalic acid was detected by its color reaction with diphenylamine (Feigl, 1966). Oxaluric acid was identified by coincidence of its R_F value on chromatograms with authentic material, by reaction with Ehrlich's reagent, and by hydrolysis in dilute HCl to oxalic acid and urea. Ribosyl- and deoxyribosylurea were located on chromatograms by their reaction with Ehrlich's reagent. They were identified by elution of these spots and hydrolysis in dilute HCl (Jones and Walker, 1963) to the corresponding sugar and urea. Urea was identified as indicated above and the sugars revealed by an ammoniacal silver nitrate spray.

Results

Nucleic Acid Components. Table I gives a summary of the kinetic results for the reaction of *m*-chloroperoxybenzoic acid with nucleic acid components. For each base, nucleoside, or nucleotide, we observed a characteristic maximum in the bell-

TABLE I: Kinetic Data for the Reaction of Nucleic Acid Components with *m*-Chloroperoxybenzoic Acid.[e]

Substrate	k_2^{max} (M^{-1} min^{-1})	pH	pK_a'
Adenine	4.7	6.2	3.9[b]
Adenosine	4.0	5.5	3.6[a]
AMP	4.6	5.8	3.55[b]
Guanosine	4.4	8.4	9.1[b]
GMP	2.8	8.5	9.4[a]
Cytosine	13.2	6.6	4.5[a]
Cytidine	4.2	6.0	4.2[a]
Deoxycytidine	4.5	6.0	4.15[b]
CMP	3.4	6.2	4.35[c]
Uracil	8.3	8.8	9.03[a]
5-Hydroxymethyluracil	11.8	8.5	9.02[a]
Uridine	3.8	8.6	9.0[b]
Deoxyuridine	3.2	8.5	9.0[d]
UMP	1.3	8.6	9.2[c]
Thymine	1.9	8.8	9.7[a]
Thymidine	0.7	8.6	9.5[b]
TMP	1.1	8.8	9.7[c]

[a] Our data, 40°, μ = 0.1. [b] Izatt and Christensen (1968). The values have been corrected to 40° using the heats of ionization given therein. [c] Jordan (1960). The values have been approximately corrected to 40° using the heats of ionization for the nucleosides. [d] Clauwaert and Stockx (1968). This value has been approximately corrected to 40° using the heat of ionization for the ribonucleoside. [e] [MCPB] = [substrate] = 3.0×10^{-3} M; [EDTA] = 1×10^{-4} M; Temperature = 40°. The pK_a' of MCPB (*m*-chloroperoxybenzoic acid) at 40° is 7.4 (Goodman *et al.*, 1962).

shaped pH–rate profile. The rate constants were corrected for the autodecomposition reaction as described in the Methods section. The rates of oxidation of thymine and its derivatives are small so that the percentage correction for the autodecomposition reaction is large. These rate constants are correspondingly less certain. Actual plots are shown for three illustrative substrates in Figure 1. Figures 2 and 3 show, respectively, typical second-order plots for cytosine and uracil and a plot of pseudo-first-order constants for cytidine and uridine *vs.* substrate concentration.

Substituent Effects. We have examined the effect of a series of substituents on the rate of reaction of *m*-chloroperoxybenzoic acid with cytosine, uracil, and less extensively, with adenosine. In order to compare these results with a known system, we have reexamined the oxidation of a few substituted pyridines as a function of pH. These results are collected in Table II. Figure 4 shows plots of the kinetic results for three of these substrates which do not show the usual bell-shaped pH–rate profile.

Ionic Strength Effects. We have examined the effect of ionic strength on the rate of reaction of both cytosine and 3-picoline with *m*-chloroperoxybenzoic acid by the addition of sodium perchlorate at constant pH. These data are given in Table III.

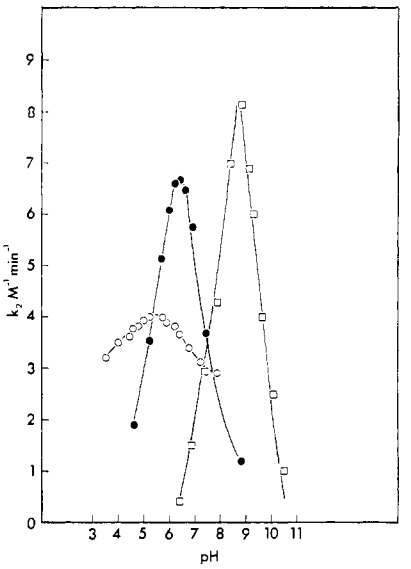

FIGURE 1: pH–rate profiles. (○) Adenosine, (●) 6-methylcytosine and (□) uracil.

Temperature Effects. The variation in the observed rate constant for three substrates together with the activation energies derived from these are given in Table IV.

Product Identification. Adenine, adenosine, 8-bromoadenosine, AMP, cytosine, 5-methylcytosine, 6-methylcytosine, 5-bromocytosine, cytidine, deoxycytidine, CMP, and the pyridines all formed products which were identified as *N*-oxides by the characteristic ultraviolet absorption spectra of spots eluted from thin-layer or paper chromatograms. For all of these compounds, with the exception of the brominated substrates, only one product corresponding to the *N*-oxide, was detected in chromatograms. For cytosine, quan-

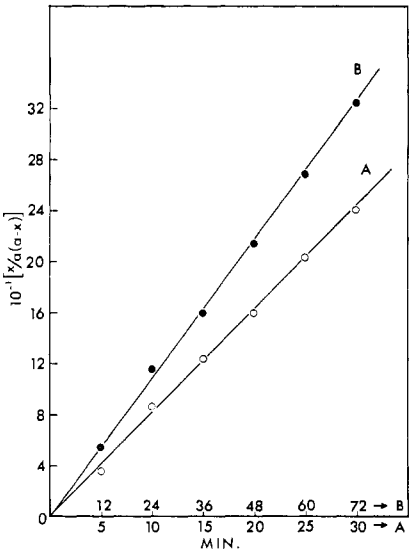

FIGURE 2: Second-order plots. (○) Cytosine (A), [cytosine] = [*m*-chloroperoxybenzoic acid] = 3×10^{-3} M, pH 5.25, 40°. (●) Uracil (B), [uracil] = [*m*-chloroperoxybenzoic acid] = 2.99×10^{-3} M, pH 9.65, 40°.

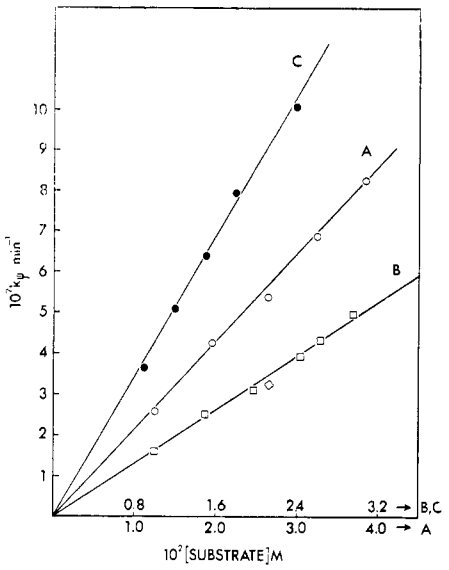

FIGURE 3: Pseudo-first-order rate constants *vs.* substrate concentration. (○) Cytidine (A), pH 4.5, 40°. (□) Uridine (B), pH 9.6, 40°. (●) Pyridine (C), pH 5.0, 40°.

TABLE II: Substituent Effects.[a]

Substrate	k_2^{max} (M^{-1} min^{-1})	pH	pK_a'
Adenosine	4.0	5.5	3.6
8-Bromoadenosine	3.0[b]	4.5[b]	3.4
Cytosine	13.2	6.6	4.5
5-Methylcytosine	4.6	6.8	4.8
6-Methylcytosine	6.7	6.4	4.8
5-Bromocytosine	32.0[b]	5.5[b]	3.0, 9.9
Uracil	8.3	8.8	9.0
5-Methyluracil	1.9	8.8	9.7
6-Methyluracil	4.3	8.6	9.3
5-Bromouracil	83.0[b]	8.4	7.8
Pyridine	8.6	6.3	5.0
3-Methylpyridine	12.5	6.6	5.5
3-Chloropyridine	1.8[b]	5.3[b]	2.8

[a] The conditions are the same as in Table I. All of the pK_a' values are our data determined at 40°, μ = 0.1 M. [b] See Discussion.

titative elution from chromatograms showed the yield of the *N*-oxide to be 90% at, pH 6.5. R_F values for the various *N*-oxides are given in Table V. Using solvent system A, spots corresponding to the *N*-oxides were eluted with 10% aqueous ammonia and the ultraviolet spectra were recorded at pH 11. The *N*-oxides from adenine, 8-bromoadenosine, adenosine, and AMP gave similar spectra with maxima around 230 and 270 mμ. The maximum at 230 mμ is indicative of the formation of the 1-*N*-oxide rather than the 7-*N*-oxide (Rhaese and Freese, 1968). Cytosine and its derivatives gave products whose ultraviolet maxima occurred around 270 mμ with a peak or shoulder around 225 mμ. The spectrum of the product from cytosine itself was identical with a sample of authentic 4-amino-2-oxopyrimidine 3-*N*-oxide. A summary of the characteristics of the ultraviolet spectra is given in Table VI. For all of these substrates, with the exception of 5-bromocytosine, tests of the reaction mixtures with Ehrlich's reagent for ureido compounds or with ammoniacal silver for free sugar were negative.

Uracil, 6-methyluracil, 5-bromouracil, 5-hydroxymethyluracil, uridine, UMP, thymine, thymidine, guanosine, and GMP all reacted with *m*-chloroperoxybenzoic acid to give ring-cleavage products. Guanine was not investigated because of its limited solubility. All of these free bases and 5-bromocytosine yielded urea as one of the products. The nucleosides gave ribosyl- or deoxyribosylurea. We have not investigated in detail the products from all of the substrates for which we have collected kinetic data. Uracil, thymine, uridine, and thymidine were investigated most thoroughly. The data for these compounds are presented below and in Table VII.

URACIL. Paper or thin-layer chromatography of reaction mixtures run at pH 8 gave three well-defined yellow spots after treatment with Ehrlich's reagent as shown in Table VII. The

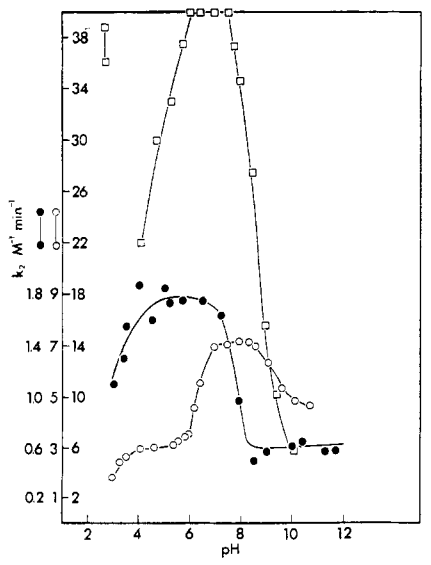

FIGURE 4: pH–rate profiles for halogenated substrates. (□) 5-Bromocytosine, (●) 3-chloropyridine, and (○) 8-bromoadenosine.

TABLE III: Effects of Ionic Strength.[a]

	A. Cytosine				
μ	0.16	0.28	0.39	0.61	0.99
k_2 (M^{-1} min^{-1})	12.9	12.6	12.2	11.9	11.3
	B. 3-Picoline				
μ	0.15	0.37	0.58	0.79	0.98
k_2 (M^{-1} min^{-1})	11.8	11.6	11.8	11.5	11.5

[a] Temperature 40°; [MCPB] = [substrate] = 3×10^{-3} M; [EDTA] = 1×10^{-4} M; pH 6.5 (cytosine) and 6.4 (3-picoline).

TABLE IV: Temperature Effects.[a]

Substrate	Temp (°C)	k_2 (M^{-1} min^{-1})	E_a (kcal/mole)
Cytosine	22	3.4	14.1
	30	6.3	
	40	13.2	
Uracil	30	3.7	14.6
	40	7.9	
	50	16.3	
3-Picoline	20	2.9	13.4
	30	6.0	
	40	11.8	

[a] [MCPB] = [substrate] = 3×10^{-3} M; [EDTA] = 1×10^{-4} M; cytosine, pH 6.5; uracil, pH 9.0; 3-picoline, pH 6.4.

component with the maximum R_F value was identified as urea. The middle component was identified as oxaluric acid. When the lowermost component was eluted with water and rechromatographed, it was found to have decomposed in part to urea and oxaluric acid. The lowermost component also reacted with 2,4-dinitrophenylhydrazine to yield a 2,4-dinitrophenylhydrazone. Formaldehyde was present following reduction of reaction mixtures with aluminum and hydrochloric acid.

THYMINE. Thymine reaction mixtures showed two yellow spots following chromatography and spraying with Ehrlich's reagent. One of these corresponded with urea. The other was a ureido derivative of low R_F value. There also appeared a blue spot following treatment with Ehrlich's reagent of R_F value close to zero. See Table VII. This same region also showed the presence of a carbonyl compound by formation of a 2,4-dinitrophenylhydrazine derivative. Reduction of the 2,4-dinitrophenylhydrazone with hydrogen and platinum oxide gave material chromatographically identical with 2-amino-1-propanol. Thymine glycol, hydrolyzed at the same pH used for the reaction of thymine with the peroxy acid, gave material which behaved similarly: chromatograms showed a spot of low R_F which formed a blue color with Ehrlich's reagent and which also gave a hydrazone among whose reduction products was 2-amino-1-propanol. No formic acid or formaldehyde was detected.

URIDINE. Uridine, as expected, followed the same pattern as uracil. Crude reaction mixtures contained two ureido derivatives and no free ribose, or urea. One of the spots corresponded to ribosylurea which, upon hydrolysis in 1 N HCl, gave ribose and urea. Formic acid was present. Deoxyuridine behaved similarly giving deoxyribosylurea.

THYMIDINE. Only one Ehrlich-positive spot was detected in reaction mixtures containing thymidine. There was no free urea. Elution and hydrolysis of this spot gave urea and deoxyribose.

GUANOSINE. Guanosine gave urea, oxalic acid, formic acid, ribosylurea, and an unidentified ultraviolet-absorbing compound with absorption maxima at 232 and 260 mμ at pH 11.

HALOGENATED SUBSTRATES. 5-Bromocytosine gave the presumed 3-*N*-oxide (Table VI). The ring-cleavage products included urea and an unidentified ureido derivative. Below pH 8.5, both the *N*-oxide and ring-cleavage products are formed with the proportion of the *N*-oxide increasing with decreasing pH. Above pH 9, only ring cleavage is observed. Delia *et al.* (1965) have reported destruction of 5-bromocytosine by *m*-chloroperoxybenzoic acid in acetic acid solvent. 8-Bromoadenosine gave the presumed 1-*N*-oxide (Table VI). This was the only product at pH 4. At pH values greater than 8, both the *N*-oxide and an unidentified compound with ultraviolet maxima at 242 and 283 mμ (pH 11) and an R_F value of 0.41 in solvent A (Table V) were detected. The proportion of the *N*-oxide decreased with increasing pH. 3-Chloropyridine gave, in addition to the expected *N*-oxide, chloride ions in the alkaline range.

TABLE V: R_F Values of Substrates and Their *N*-Oxides.[a]

Compounds	R_F Values Solvent A	Solvent B	Solvent C
Adenine	0.76	0.40	0.53
Adenine 1-*N*-oxide	0.62	0.50	0.25
Adenosine	0.74	0.52	0.39
Adenosine 1-*N*-oxide	0.58	0.69	0.15
8-Bromoadenosine	0.75		
8-Bromoadenosine 1-*N*-oxide	0.58		
AMP	0.58		
AMP-1-*N*-oxide	0.47		
Cytosine	0.70	0.70	0.34
Cytosine 3-*N*-oxide	0.51	0.65	0.23
Cytidine	0.71	0.78	
Cytidine 3-*N*-oxide	0.49	0.83	
CMP	0.52		
CMP-3-*N*-oxide	0.38		
Deoxycytidine	0.65		
Deoxycytidine 3-*N*-oxide	0.46		
5-Methylcytosine	0.65	0.70	0.39
5-Methylcytosine 3-*N*-oxide	0.61	0.75	0.15
6-Methylcytosine	0.71		
6-Methylcytosine 3-*N*-oxide	0.51		
5-Bromocytosine	0.60		
5-Bromocytosine 3-*N*-oxide	0.45		
Guanosine	0.66	0.66	0.19
Guanosine *N*-oxide (?)	0.51	0.62	

[a] Ascending chromatography on No. 1 Whatman paper in the machine cut direction. Solvent A, 2-propanol–1% ammonium sulfate (1:2, v/v); solvent B, isoamyl alcohol–5% Na_2HPO_4 (3:2, v/v); and solvent C, 1-butanol saturated with 15% urea solution.

Discussion

The pH–rate profiles for the reactions of nucleic acid components with *m*-chloroperoxybenzoic acid are all bell-shaped curves with maxima which fall into one of two groups: cytosine, adenine, and their derivatives show maxima on the acid

TABLE VI: Ultraviolet Maxima for the *N*-Oxides.

Compound	max (mμ)	Peak Ratios[a]
Cytosine 3-*N*-oxide	221, 289	0.21
5-Methylcytosine 3-*N*-oxide	220, 292	0.23
6-Methylcytosine 3-*N*-oxide	220, 288	0.24
5-Bromocytosine 3-*N*-oxide	215, 259	0.32
Cytidine 3-*N*-oxide	225 (S), 270	0.43
CMP-3-*N*-oxide	225 (S), 272	0.29
Adenine 1-*N*-oxide	232, 274	0.17
Adenosine 1-*N*-oxide	232, 265	0.34
8-Bromoadenosine 1-*N*-oxide	236, 265	0.34
AMP-1-*N*-oxide	232, 262	0.23
Guanosine *N*-oxide (?)	232, 260	0.88

[a] Optical density long-wavelength peak/optical density short-wavelength peak or shoulder (S); pH 11.

TABLE VII: R_F Values of the Oxidation Products of Uracil and Thymine.

Compounds	Solvent A[a]	Solvent B[b]	Solvent C[c]
Uracil	0.70	0.52	0.53[d]
Urea[e]	0.79	0.38	0.49[d]
Oxaluric acid[e]	0.47	0.15	0.29[d]
Uracil reaction mixture			
Spot A[e]	0.78	0.37	0.49[d]
Spot B[e]	0.46	0.15	0.26[d]
Spot C[e]	0.32	0.05	0.11[d]
Thymine	0.70	0.73	
Thymine reaction mixture			
Spot A[e]	0.59[d]	0.40	
Spot B[e]	0.31[d]	0.18	
Spot C		0.02[f]	

[a] Solvent A, 1-butanol–acetic acid–water (2:1:1, v/v). [b] Solvent B, 1-butanol–ethanol–water (4:1:5, v/v). [c] Solvent C, *sec*-butyl alcohol–water (5:7, v/v). [d] These values are for thin-layer chromatography on cellulose. Other data are for chromatography on Whatman No. 1 paper. [e] Ehrlich's reagent positive. [f] Blue with Ehrlich's Reagent, see text.

side of the pK_a of the peroxy acid in the vicinity of pH 6, while uracil, thymine, guanosine, and their derivatives show maxima on the alkaline side in the vicinity of pH 8. The former group involves substrates which ionize to form the neutral substrate by loss of a proton with a pK_a around pH 4.5, while the latter group involves substrates which ionize to form anions by loss of a proton with pK_a values around 9.5. The kinetic results in each case may be formally accounted for by a pair of mechanisms: for the first group either by reaction of the substrate cation with the peroxy acid anion, or by reaction of the neutral substrate with the un-ionized peroxy acid. For the second group the observed pH–rate profile may be accounted for by reaction of the peroxy acid anion with the neutral substrate or by reaction of the anionic substrate with the un-ionized peroxy acid. We can choose the predominant mechanism in each case on the basis of substrate substituent effects. Thus, for cytosine and uracil, electron-withdrawing groups on the substrate increase the rate so that one may characterize the reactions as attack of the peroxy acid anion on the cationic substrate for cytosine and on the neutral substrate for uracil. For adenine, since the substituent effect is in the opposite direction to that for cytosine, we view the reaction as predominantly attack by the un-ionized peroxy acid on the neutral substrate. The observed ionic strength effects are in accord with the idea that the cytosine case is a reaction between oppositely charged species: the rate decreases with an increase in ionic strength, whereas for the pyridine system, there is almost no effect.

The substituent effects for the case of the nucleosides require further comment. The pK_a values for the nucleosides are uniformly lower than the values for the free bases showing the sugar residue to be electron withdrawing. Considering the mechanisms which we have postulated, we would expect the rates for cytidine, uridine, and thymidine to be greater than the rates for the free bases. Because we find the opposite effect,we invoke steric hindrance, since, in each case, we have supposed on other grounds that rate-limiting attack occurs at the carbon *ortho* to the nitrogen bearing the sugar residue.

For all of these substrates, the observed second-order rate constant, k_2, is a pH-dependent value related to the true rate constant, k, by the relationship

$$k_2 = \frac{k\mathrm{C}[\mathrm{H}^+]}{[\mathrm{H}^+]^2 + [\mathrm{H}^+]K_1 + [\mathrm{H}^+]K_2 + K_1K_2}$$

where C is either K_1 or K_2 (the ionization constants for the peroxy acid and the substrate, depending upon which ionized species are the actual reactants). It is, of course, possible, with the use of these equations, to calculate absolute rate constants and hence entropies of activation, for the reactions in which we have measured the temperature dependence. We have not formally reported these because of the possibility that two types of reactions might well be occurring together. Using cytosine as an example, all we can say is that the reaction between the cytosine cation and the peroxy acid anion predominates over the reaction between the un-ionized peroxy acid and the neutral amine in the sense that the product of the Hammett ρ value for the reaction and the percentage of the reaction going by the former pathway is larger than the corresponding product for the latter pathway. Without knowledge of the ρ values involved, we cannot specifiy which pathway is responsible for the greater proportion of the product.

In chemical terms, the reaction of adenine as well as pyridine with *m*-chloroperoxybenzoic acid may be simply formulated as the expected electrophilic attack at nitrogen and oxygen transfer as shown in Figure 5a (Ochiai, 1967; Modena and Todesco, 1960; Dondoni *et al.*, 1961). For cytosine, on the other hand, the predominant course of the reaction is nucleophilic attack by the peroxy acid anion on the cationic substrate. The site of the initial attack is not certain, but one possibility is at the carbonyl group followed by a fast rearrangement as shown in Figure 5b. This unexpected result is of con-

siderable interest in nucleic acid chemistry, since it means that substituent effects in the cytosine and adenine systems run in opposite directions: substituents which increase the rate of *N*-oxide formation in the cytosine system decrease the rate in the adenine case and *vice versa*. The generality of *N*-oxide formation by nucleophilic attack on structures of the cytosine type has yet to be examined.

Uracil (and thymine) are systems containing the grouping COCH=CH. Reactions of peroxides with systems of this general type have been recently reviewed (Lee and Uff, 1967). Our data for uracil and its derivatives point to reaction of the anion of the peroxy acid with the neutral substrate. We can rationalize our findings on the basis of the schemes set out in Figure 5c,d. In each case, we visualize attack at the 5,6-double bond, epoxide formation, hydrolysis to the glycol, and then subsequent reactions following the hydrolytic scheme proposed by Jones and his coworkers who have carefully investigated the permanganate oxidation of most of these substrates (Benn *et al.*, 1960; Chatamra and Jones, 1963; Howgate *et al.*, 1968). The observations of Priess and Zillig (1965) are consistent with an analogous reaction involving the anion of hydrogen peroxide.

In addition to *N*-oxide formation, 3-chloropyridine undergoes an additional mode of reaction. Chloride ions are produced in the basic range. We interpret this pH–rate profile as the sum of a bell-shaped curve for electrophilic attack at nitrogen to yield the *N*-oxide (the peroxy acid and the neutral substrate) with k_{max} = 1.8 M^{-1} min^{-1} at pH 5.3 and an S-shaped curve with $^1/_2k_{max}$ = 0.3 M^{-1} min^{-1} at pH 7.6 representing displacement of the chloride ion from the neutral substrate by the peroxyanion.

The data for the three brominated substrates are complicated, particularly in the basic range, by the possibility of bromide displacement by the peroxy anion and subsequent oxidation to bromine by the peroxy acid. For 5-bromocytosine and 8-bromoadenosine, our interest was to define sufficiently the rate constant for the reaction leading to *N*-oxide formation. For the case of 8-bromoadenosine, we can see a shoulder in the pH–rate profile in the vicinity of pH 4.5. Only *N*-oxide formation was detected in this pH range. Since the other reactions leading to peroxide disappearance (presumably bromide displacement and subsequent oxidation) could only increase the apparent value for the rate of oxidation of 8-bromoadenosine to the *N*-oxide and since the observed value for k_{max} in this range is below that for adenosine, we feel justified in our conclusion that the substitution of bromine results in a decrease in the rate of *N*-oxide formation.

We view the data for 5-bromocytosine as an indication that bromine substitution results in a more rapid rate of *N*-oxide formation than for cytosine itself. This view is in line with the data for the methyl-substituted cytosines. The data for 5-bromocytosine may be formally decomposed into two bell-shaped curves, one for nucleophilic attack at the 5,6-double bond leading to ring-cleavage products (the peroxyanion and the neutral substrate) with k_{max} = 30 ± 3M^{-1} min^{-1} at pH 8.5, and one for nucleophilic attack of the peroxy anion on the cationic substrate leading to *N*-oxide formation with k_{max} = 32 ± 3 M^{-1} min^{-1} at pH 5.5. The fact that at least two reactions are occurring makes these conclusions less certain, so that it is possible, though we think it unlikely, that our estimate of the rate constant for *N*-oxide formation is so far wrong as to bring it below the value for cytosine. Similarly,

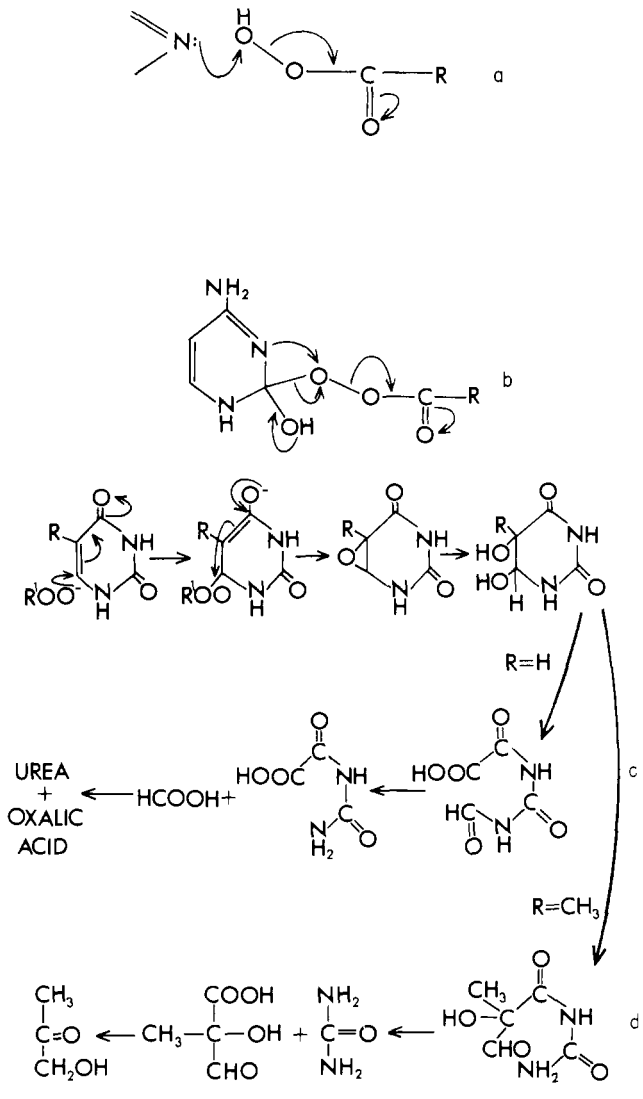

FIGURE 5

for 5-bromouracil, the value given in Table II for k_2 is a maximum. The minimum value, assuming rapid oxidation of displaced bromide, is 33 M^{-1} min^{-1}.

References

Albert, A., and Serjeant, E. P. (1962), Ionization Constants of Acids and Bases, London, Methuen, Chapter 2.

Arantz, B. W., and Brown, D. J. (1968), *in* Synthetic Procedures in Nucleic Acid Chemistry, Vol. 1, Zorbach, W. W., and Tipson, R. S., Ed., New York, N. Y., Interscience, p 55.

Ball, D. L., and Edwards, J. O. (1956), *J. Am. Chem. Soc. 78*, 1125.

Ball, D. L., and Edwards, J. O. (1958), *J. Phys. Chem. 62*, 343.

Baudisch, O., and Davidson, D. (1925), *J. Biol. Chem. 64*, 233.

Beaven, G. H., Holiday, E. R., and Johnson, E. A. (1955), *in* The Nucleic Acids, Chargaff, E., and Davidson, J. N., Ed., New York, N. Y., Academic, p 493 ff.

Behrman, E. J., and Edwards, J. O. (1967), *Progr. Phys. Org. Chem. 4*, 93.
Benn, M. H., Chatamra, B., and Jones, A. S. (1960), *J. Chem. Soc.*, 1014.
Brown, D. J. (1950), *J. Soc. Chem. Ind. (London) 69*, 353.
Chatamra, B., and Jones, A. S. (1963), *J. Chem. Soc.*, 811.
Clauwaert, J., and Stockx, J. (1968), *Z. Naturforsch. B23*, 25.
Cramer, F., Randerath, K., and Schäfer, E. A. (1963), *Biochim. Biophys. Acta 72*, 150.
Cramer, F., and Seidel, H. (1963), *Biochim. Biophys. Acta 72*, 157.
Curci, R., and Modena, G. (1965), *Tetrahedron 22*, 1227.
Delia, T. J., and Brown, G. B. (1966), *J. Org. Chem. 31*, 178.
Delia, T. J., Olsen, M. J., and Brown, G. B. (1965), *J. Org. Chem. 30*, 2766.
Dondoni, A., Modena, G., and Todesco, P. E. (1961), *Gazz. Chim. Ital. 91*, 613.
Feigl, F. (1966), Spot Tests in Organic Analysis, New York, N. Y., Elsevier, pp 451, 457.
Fink, R. M., Cline, R. E., McGaughey, C., and Fink, K. (1956), *Anal. Chem. 28*, 4.
Goodman, J. F., Robson, P., and Wilson, E. R. (1962), *Trans. Faraday Soc. 58*, 1846.
Howgate, P., Jones, A. S., and Tittensor, J. R. (1968), *J. Chem. Soc. C*, 275.
Izatt, R. M., and Christensen, J. J. (1968), in *Handbook of Bio-Chemistry*, Sober, H. A., Ed., Cleveland, Chemical Rubber, pp J49ff.
Johns, C. O. (1908), *Am. Chem. J. 40*, 348.
Jones, A. S., and Walker, R. T. (1963), *J. Chem. Soc.*, 3556.
Jordan, D. O. (1960), The Chemistry of Nucleic Acids, Washington, Butterworths, p 134 ff.
Klötzer, W. (1965), *Monatsh. Chem. 96*, 169.
Lee, J. B., and Uff, B. C. (1967), *Quart. Rev. (London)* 429.
McCormick, D. B. (1966), *Biochemistry 5*, 746.
Modena, G., and Todesco, P. E. (1960), *Gazz. Chim. Ital. 90*, 702.
Ochiai, E. (1967), Aromatic Amine Oxides, New York, N. Y., Elsevier.
Polonovski, M., Pesson, M., and Schmitt, H. (1948), *Bull. Soc. Chim. France 15*, 392.
Priess, H., and Zillig, W. (1965), *Z. Physiol. Chem. 342*, 73.
Rhaese, H. J., and Freese, E. (1968), *Biochim. Biophys. Acta 155*, 476.
Rhaese, H. J., Freese, E., and Melzer, M. S. (1968), *Biochim. Biophys. Acta 155*, 491.
Ross, V. F., and Edwards, J. O. (1967), *in* The Chemistry of Boron and Its Compounds, Muetterties, E. L., Ed., New York, N. Y., Wiley, p 201.
Russell, G. A. (1961), *in* Investigations of Rates and Mechanisms of Reactions, Part 1, Friess, S. L., Lewis, E. S., and Weissberger, A., Ed., 2nd ed, New York, N. Y., Interscience, p 343.
Schwartz, N. N., and Blumbergs, J. H. (1964), *J. Org. Chem. 29*, 1976.
Seidel, H. (1967), *Biochim. Biophys. Acta 138*, 98.
Sigel, H., and Brintzinger, H. (1965), *Helv. Chim. Acta 48*, 433.
Stevens, M. A., Magrath, D. I., Smith, H. W., and Brown, G. B. (1958), *J. Am. Chem. Soc. 80*, 2755, 2759.
Stevens, M. A., Smith, H. W., and Brown, G. B. (1959), *J. Am. Chem. Soc. 81*, 1734.
Subbaraman, L. R., Subbaraman, J., and Behrman, E. J. (1968), *Chem. Commun.*, 1024, 1268.