REACTIONS BETWEEN SINGLET OXYGEN AND THE CONSTITUENTS OF NUCLEIC ACIDS

IMPORTANCE OF REACTIONS IN PHOTODYNAMIC PROCESSES

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ABSTRACT Bases, nucleosides, nucleotides, and polynucleotides were exposed to chemically generated singlet oxygen to determine whether the species oxidized paralleled those oxidized in photodynamic reactions. In neutral or basic aqueous solution guanine, guanosine, deoxyguanosine, guanylic acid, deoxyguanylic acid, thymine, and uracil reacted with singlet oxygen. Since these compounds are oxidized in photodynamic processes, this study provides further evidence that singlet oxygen is the active intermediate in the photodynamic oxidation of nucleic acid constituents. Dienophilic attack by singlet oxygen is considered to be a plausible mechanism in these reactions.

INTRODUCTION

Recently Foote (1968), Kearns and Khan (1969), and others have suggested that singlet oxygen may be the active intermediate in some of the so-called photodynamic reactions. These reactions are generally considered to refer to photosensitized oxygenation processes and may be represented by the following scheme:

$$R + O_2 \xrightarrow[S]{\text{visible light}} P,$$

where R represents the substrate molecule, S represents the sensitizer molecule, and P represents the product molecule(s).

The photodynamic oxidation of nucleic acids and their components has been extensively studied by several workers (see Table I). Of all the nucleotides investigated, the guanine derivatives alone were appreciably oxidized although slight effects were noticed in studies of thymidine and thymidylic acid. The free bases uracil and thymine

TABLE I

THE PHOTODYNAMIC OXIDATION OF NUCLEIC ACID CONSTITUENTS

Sensitizer	Nucleic acid components oxidized*	Reference
Methylene blue	G, dGMP, T, GS, TMP, U, X	Simon and Van V unakis (1962, 1964) Waskell, Sastry, and Gordon (1966)
Acridine orange	GS	Sastry and Gordon (1966)
Lumichrome	dGMP, GMP, GS, G	Sussenbach and Berends (1963)
Thiopyronine	G	Lochmann, Stein, and Haefner (1964)
Toluidine blue	dGMP, GMP, GS, G	Simon and Van Vunakis (1964)
Eosin Y	dGMP, GMP, GS, G	Simon and Van Vunakis (1964)
Rose bengal	dGMP, GMP, GS, G	Simon and Van Vunakis (1964)
Thionine	dGMP, GMP, GS, G	Simon and Van Vunakis (1964)
Riboflavin	dGMP, GMP, GS, G	Simon and Van Vunakis (1964)

* G = guanine, GS = guanosine, GMP = guanylic acid, dGMP = deoxyguanylic acid, T = thymine, TMP = thymidylic acid, U = uracil, X = xanthosine

are photooxidized, but not to the extent found for guanine. The purpose of this research was to determine whether singlet oxygen, chemically generated by the hydrogen peroxide–sodium hypochlorite reaction (Brown and Ogryzlo, 1964; Khan and Kasha, 1966), has the same specificity of reaction with nucleic acid constituents. We believe that this work is the first study of reactions between the common nucleic acid constituents and chemically generated singlet oxygen.

EXPERIMENTAL

The experimental technique employed in this study has been adapted from that used by Foote, Wexler, Ando, and Higgins (1968). Solutions of substrate and hydrogen peroxide were combined at 0°C and stirred rapidly at this temperature. The sodium hypochlorite was then added to this mixture drop by drop. While low concentrations of hydrogen peroxide lead to higher yields of singlet oxygen (Foote et al., 1968), it was found that fairly high concentrations of peroxide were necessary to prevent significant reaction between the substrate and sodium hypochlorite. The technique requires that essentially no reaction take place between the substrate and hydrogen peroxide. Fortunately, most of the bases and nucleosides were insensitive to peroxide under the conditions used. While the nucleotides and polynucleotides were degraded by hydrogen peroxide in neutral or slightly acidic solution, they were completely unaffected at pH's greater than ten. This implicated the formation of the reactive peroxy phosphate species at lower pH. For this reason nucleotides were studied in basic solution only.

The dropwise addition of sodium hypochlorite led to a gradual increase in the pH of the reaction mixture. It was impossible, therefore, to characterize the pH of the reaction other than to state that the reactions were carried out in neutral solution (pH 6.0–8.5) and in basic solution (pH $>$ 10).

A typical experiment is as follows. 4 ml of an aqueous 2.5×10^{-3} M solution of substrate were cooled to 0°C. 10 ml cold water (or 9.5 ml water and 0.5 ml 1 N NaOH) and 8 ml of 8.5 M (30%) hydrogen peroxide were added. This mixture was maintained at 0°C and vigorously agitated with a magnetic stirrer while 18 ml of 0.7 M sodium hypochlorite were added

dropwise over a 60 min period. After dilution to a substrate concentration of 6.2×10^{-6} M, the excess hydrogen peroxide was removed by catalysis with manganese dioxide.

In experiments at pH 6.0–8.5, a few substrates had to be used in lower concentration because of their lower solubility. In these cases the excess hydrogen peroxide remaining after the reaction could not be diluted prior to its removal by catalysis with manganese dioxide. Care was taken to insure that this reaction was not too vigorous.

The effects of hydrogen peroxide alone on the substrate were evaluated by exposing a sample of substrate to a concentration of peroxide identical to that used in the reaction mixture for 60 min at 0°C. After all traces of peroxide had been removed, the ultraviolet (UV) spectra of the reaction sample and the peroxide control were compared with the spectrum of an untreated solution of 6.2×10^{-6} M substrate.

The effects of sodium hypochlorite on the substrate were studied in separate experiments in which a 2.5×10^{-4} M solution of the substrate was exposed to a hypochlorite concentration of 2.3×10^{-3} M. This was the highest concentration of hypochlorite that could be used, because its ultraviolet absorption at 290 nm interfered with the analysis of most substrates. Since the effective hypochlorite concentration in the reaction mixture is unknown, this control serves only to give the relative reactivity of the various substrates with hypochlorite.

RESULTS

The percentages of substrate remaining after exposure to singlet oxygen plus the pertinent peroxide and hypochlorite controls have been tabulated in Tables II, III, IV, and V. The values are believed to be correct to approximately ±5%. Thus, when the per cent substrate remaining was 10% less than the value of the peroxide control, it was judged that a reaction with singlet oxygen had occurred. There are two exceptions to this statement. In the case of guanine in neutral solution, the as-

TABLE II

EXPERIMENTAL RESULTS, BASES

Biological molecule	Conditions (number of runs)	Column I % remaining $^1O_2$ treatment	Column II % remaining H_2O_2 treatment	Column III % remaining NaOCl treatment	Column IV $^1O_2$ reaction?
Adenine	H_2O (2)	75	60	65	
"	H_2O + NaOH (1)	83	87	100	
Cytosine	H_2O (2)	92	88	69	
"	H_2O + NaOH (1)	89	89	100	
Guanine	H_2O (3)	<43	48	—	Yes
"	H_2O + NaOH (2)	<54	100	Shift	Yes
Uracil	H_2O (2)	<55	92	Shift	Yes
"	H_2O + NaOH (1)	<57	62	100	Yes
Thymine	H_2O (2)	64	92	19	Yes
"	H_2O + NaOH (1)	59	82	100	Yes

TABLE III

EXPERIMENTAL RESULTS, NUCLEOSIDES

Biological molecule	Conditions (number of runs)	Column I % remaining $^1O_2$ treatment	Column II % remaining H_2O_2 treatment	Column III % remaining NaOCl treatment	Column IV $^1O_2$ reaction?
Adenosine	H_2O (2)	92	93	26	
"	H_2O + NaOH (1)	95	94	100	
Cytidine	H_2O (1)	84	84	88	
"	H_2O + NaOH (1)	93	92	100	
Guanosine	H_2O (3)	<58	94	30	Yes
"	H_2O + NaOH (2)	<68	94	100	Yes
Uridine	H_2O (2)	80	84	Shift	
"	H_2O + NaOH (1)	74	42	100	
Thymidine	H_2O (2)	87	95	Shift	
"	H_2O + NaOH (1)	87	88	100	
Deoxyadenosine	H_2O (1)	94	96	29	
"	H_2O + NaOH (1)	95	100	100	
Deoxycytidine	H_2O (1)	94	94	<34	
"	H_2O + NaOH (1)	93	97	100	
Deoxyguanosine	H_2O (2)	<66	100	<30	Yes
"	H_2O + NaOH (1)	<70	94	100	Yes
Xanthosine	H_2O (1)	71	86	<38	Yes
"	H_2O + NaOH (1)	76	100	96	Yes

signed error is many times larger because of its adsorption to both manganese dioxide and the container. A reaction between guanine and singlet oxygen was judged to occur since the shape of the guanine absorption was altered considerably. Uracil in basic solution was another special case. Here a reaction product exhibited a UV absorption near the uracil maximum at 284 nm, adding to the measured peak height. In all cases where the product absorption appeared beneath the substrate absorption, the symbol < has been used to indicate that the value listed is probably several per cent too high. When a shift has been inserted it means that the absorption peak shifted in a manner that could not be accounted for by pH changes.

The peroxide control illustrates the relative sensitivity of the substrates to a peroxide concentration equal to that used in the reaction mixture. Most substrates were quite resistant to peroxide in neutral solution with the exception of adenine,

TABLE IV

EXPERIMENTAL RESULTS, NUCLEOTIDES AND POLYNUCLEOTIDES

Biological molecule	Conditions (number of runs)	Column I % remaining $^1O_2$ treatment	Column II % remaining H_2O_2 treatment	Column III % remaining NaOCl treatment	Column IV $^1O_2$ reaction?
Adenylic acid	H_2O + NaOH (1)	95	99	100	
Cytidylic acid	H_2O + NaOH (1)	96	100	100	
Guanylic acid	H_2O + NaOH (1)	64	94	100	Yes
Uridylic acid	H_2O + NaOH (1)	86	88	100	
Thymidylic acid	H_2O + NaOH (1)	96	97	100	
Deoxyadenylic acid	H_2O + NaOH (1)	94	100	100	
Deoxycytidylic acid	H_2O + NaOH (1)	95	98	100	
Deoxyguanylic acid	H_2O + NaOH (1)	64	98	90	Yes
Polyadenylic acid	H_2O + NaOH (1)	93	88	100	
Polyuridylic acid	H_2O + NaOH (1)	89	89	100	
Polycytidylic acid *	H_2O + NaOH (1)	88	95	100	

* Polyguanylic acid was not available from the supplier.

TABLE V

EXPERIMENTAL RESULTS, MODEL PURINES

Purine	Conditions (number of runs)	Column I % remaining $^1O_2$ treatment	Column II % remaining H_2O_2 treatment	Column III % remaining NaOCl treatment	Column IV $^1O_2$ reaction?
8-azaguanine	H_2O (1)	81	81	Shift	
"	H_2O + NaOH (2)	82	99	96	Yes
8-bromoguanosine	H_2O (1)	75	91	Shift	Yes
"	H_2O + NaOH (1)	75	96	100	Yes
8-azaxanthine	H_2O + NaOH (2)	81	100	100	Yes
2,6-diaminopurine	H_2O (1)	85	100	Shift	Yes
"	H_2O + NaOH (2)	78	100	100	Yes
8-aza-2,6-diamino-purine	H_2O (1)	95	94	54	
"	H_2O + NaOH (2)	96	100	100	

8-azaguanine, and the nucleotides. Uracil and uridine were peculiar in that addition of base increased their reaction with peroxide, whereas addition of base usually served to stabilize the molecules against peroxide attack. The substrates in neutral solution were usually sensitive to sodium hypochlorite. In basic solution, however,

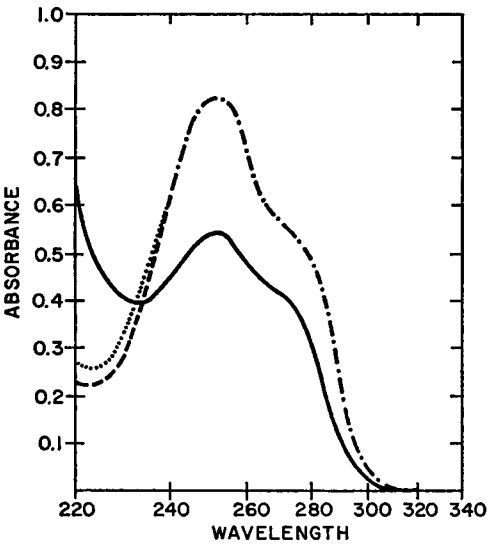


FIGURE 1

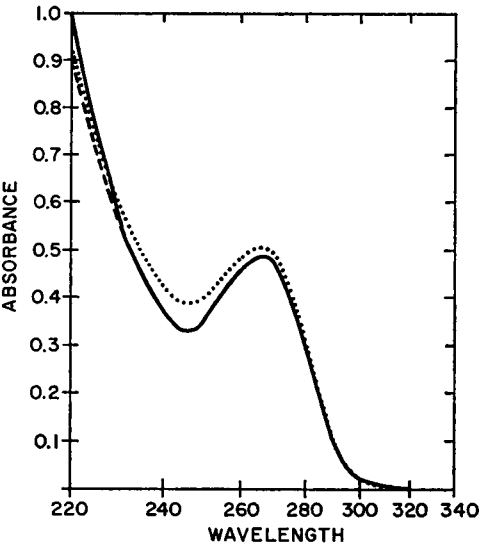


FIGURE 2

FIGURE 1 Ultraviolet spectra: ..... guanosine, ------ guanosine treated with hydrogen peroxide, ——— guanosine treated with singlet oxygen.

FIGURE 2 Ultraviolet spectra: ..... thymidylic acid, ------ thymidylic acid treated with hydrogen peroxide, ——— thymidylic acid treated with singlet oxygen.

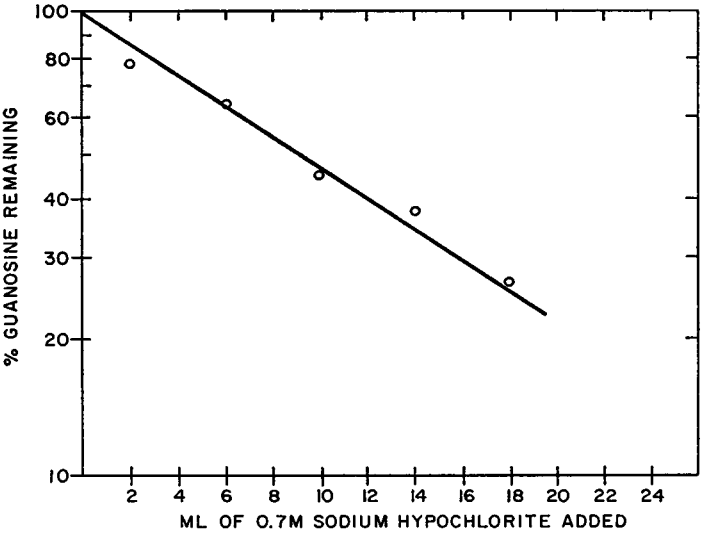


FIGURE 3 Destruction of guanosine by singlet oxygen in neutral solution. Points were estimated from peak-to-trough ratios of the UV spectra.

they became very resistant and guanine alone exhibited any appreciable reaction. Since the results in Column I of Tables II–V cannot be explained on the basis of either of these controls, then singlet oxygen must be the reacting species.

Figs. 1 and 2 show the spectra obtained for two substances, deoxyguanosine which

reacted with singlet oxygen and thymidylic acid which did not react with singlet oxygen. The spectrum of the reacted substrate was altered at shorter wavelengths in all cases studied. Runs in which no substrate was present demonstrated that the peroxide-hypochlorite reaction produced an unidentified species that absorbed in this region. Fig. 3 gives the per cent guanosine remaining as a function of the amount of sodium hypochlorite added to the reaction mixture. The damage to the molecule is first order according to the relationship

$$\frac{dx}{dy} = -kx,$$

where x is the concentration of guanosine, y is the amount of sodium hypochlorite added, and k is a constant.

DISCUSSION

The results of this singlet oxygen work and the work of others on photodynamic oxidations of naturally occurring nucleic acid components are compared in Table VI. The only inconsistencies are found for the thymidine and thymidylic acid mole-

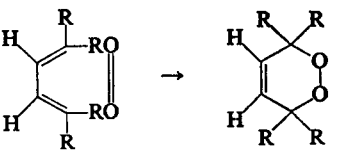
TABLE VI

COMPARISON OF SINGLET OXYGEN AND PHOTODYNAMIC DAMAGE TO COMMON NUCLEIC ACID CONSTITUENTS

Nucleic acid constituent	Damaged by $^1O_2$?	Damaged by dye, $h\nu$, and O_2?
Adenine	No	No
Cytidine	No	No
Guanine	Yes	Yes
Uracil	Yes	Yes
Thymine	Yes	Yes
Adenosine	No	No
Deoxyadenosine	No	No
Cytidine	No	No
Deoxycytidine	No	No
Guanosine	Yes	Yes
Deoxyguanosine	Yes	Yes
Uridine	No	No
Thymidine	No	Slight
Xanthosine	Yes	Yes
Adenylic acid	No	No
Deoxyadenylic acid	No	No
Cytidylic acid	No	No
Deoxycytidylic acid	No	No
Guanylic acid	Yes	Yes
Deoxyguanylic acid	Yes	Yes
Uridylic acid	No	No
Thymidylic acid	No	Slight

cules. Simon and Van Vunakis (1962) report a small photodynamic reaction with both of these species. Thus, for the commonly observed nucleic acid components, the singlet oxygen studies correspond extremely well with the results obtained in photodynamic studies. It is likely, therefore, that the principle active intermediate in the photodynamic oxidation of the compounds studied here is singlet oxygen.

Considerable progress has been made in elucidating the ways in which singlet oxygen reacts with many types of organic compounds. An excellent review of this subject has been given by Gollnick (1968). However, comparatively little is known about the ways in which singlet oxygen might react with purine and pyrimidine residues. Singlet oxygen has been shown to add across dienes (Scheme I) and unsaturated ring compounds, and it is possible that it may react similarly with purine and pyrimidine systems.



SCHEME I

Some evidence (Kearns and Khan, 1969) indicates that the dienophilic property illustrated in Scheme I is a characteristic of singlet oxygen, in the $^1\Delta_g$ state. The other state of singlet oxygen, $^1\Sigma_g$, is thought to react via hydrogen abstraction reactions. Since both states of singlet oxygen may be produced in some photodynamic reactions, we did not wish to eliminate either of the possibilities, and no radical inhibitors or buffers were used in this study. The radical mechanism does not appear to explain the specificity of singlet oxygen attack observed in these experiments. This may be due to the short lifetime of the $^1\Sigma_g$ state compared to the $^1\Delta_g$ state or to a lack of substrates which are good hydrogen atom donors.

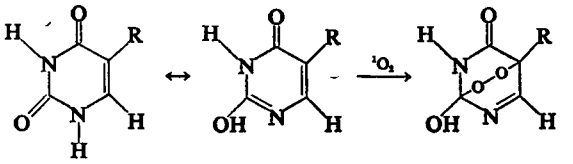
Since the oxygen molecule is an electrophilic species it is likely that enhancement of the dienophilic reaction (Scheme I) should occur if one or both of the terminal carbons carry a net negative charge. In fact it may be that the presence of these terminal charges is necessary for the reaction to proceed at an observable rate. If the terminal atom is nitrogen, the cycloaddition reaction should be inhibited because nitrogen and oxygen would compete for the negative charge.

In guanine, if such a cycloaddition reaction occurs, singlet oxygen may add across the 1–4 positions, the 2–5 positions, the 3–6 positions, the 4–8 positions, and the 5–8 positions. The 5–8 addition is not possible in guanosine or guanylic acid. The 1–4 and 3–6 additions can be eliminated since one of the terminal atoms is a nitrogen in each case. Recent studies indicate that addition may occur across the imidazole ring, that is, 4–8 or 5–8 addition. Zenda, Saneyoshi, and Chihara (1965) and Fried-

man (1968) have found that while guanine, xanthine, and 2,6-diaminopurine can be readily photooxygenated, 8-aza–derivatives of these molecules can not. Matsuura and Saito (1967, 1968, 1969) have found that the photosensitized oxygenation of xanthine and 1,3-dimethylxanthine leads to products that can best be explained by singlet oxygen addition across the imidazole part of the molecule. Furthermore, all the reaction products that contain the 8-carbon atom have been found to be oxidized at this position. This includes reaction products from guanine (Sussenbach and Berends, 1963), guanosine (Waskell, Sastry, and Gordon, 1966), xanthine (Matsuura and Saito 1968), and theophylline (Simon and Van Vunakis, 1964). Friedman (1968) has attempted to further elucidate the mechanism by studying the photooxygenation of C2 and C8 labelled uric acid. The recent work of Matsuura and Saito (1968, 1969) suggests, however, that the oxygenation of uric acid does not proceed via a cycloaddition reaction.

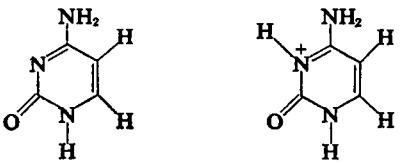
Some other studies suggest that 2–5 addition across the pyrimidine ring of the guanine molecule cannot be eliminated. The presence of a substituent on the 2-position of the purine ring determines the sensitivity of the system to singlet oxygen. Guanine, 2,6-diaminopurine, and 2-aminopurine react readily (Wacker, Dellweg, Trager, Kornhauser, Lodemann, Turck, Selzer, Chandra, and Ishimoto, 1964) whereas adenine is one of the most resistant purines. The presence or absence of a substituent on the 8-position has little effect on the sensitivity of the molecule (Zenda, Saneyoshi, and Chihara, 1965) although we have observed that 8-bromoguanosine may be slightly less sensitive than guanosine to chemically generated singlet oxygen. We have also studied the effects of chemically generated singlet oxygen on the 8-aza–derivatives of guanine, xanthine, and 2,6-diaminopurine. 8-aza-guanine does not react at neutral pH, but does react to a moderate extent at pH's greater than 10. 8-aza-xanthine could not be studied in neutral solution because of its reaction with peroxide, but it too reacted moderately with singlet oxygen at pH's greater than 10. 8-aza-2,6-diaminopurine was resistant to singlet oxygen in both neutral and basic solution. The latter result is not surprising in that 2,6-diaminopurine was less sensitive to attack by singlet oxgyen than was either guanine or xanthine species, and consequently the even more resistant 8-aza–form may not have reacted sufficiently in base to be detected by our method. Nevertheless, singlet oxygen may react under the appropriate conditions with purine derivatives having a nitrogen atom at the 8-position although the reactions are somewhat less efficient than those observed with derivatives having a carbon atom at the 8-position.

Further evidence that singlet oxygen can react with pyrimidine systems is gained from the observation that thymine and uracil are oxidized both photodynamically and with the peroxide-hypochlorite reaction. Even though thymine and uracil were sensitive to attack by singlet oxygen, we could not detect a reaction between singlet oxygen and thymidine, uridine, thymidylic acid, or uridylic acid. One explanation of this requires that a small amount of a tautomeric form of the pyrimidine is present in the solution.



R = H, uracil
R = CH_3, thymine

This mechanism is appealing because the nucleosides and nucleotides of thymine and uracil do not have this tautomeric form and therefore should not react. However, cytosine should be just as reactive provided that it has the same tautomeric form. While nuclear magnetic resonance studies have shown that uracil and thymine are primarily in the diketo form (Kokko, Goldstein, and Mandell, 1961) and that cytosine exists as tautomeric forms I and II (Katritzky and Waring, 1963), they do not estimate which, if any, of the other tautomers are present at low concentration. A study of various substituted pyrimidines may test the validity of the mechanism.



I II

In conclusion, it should be pointed out that singlet oxygen is probably the active intermediate in the photodynamic oxygenations of the nucleic acid components studied here. The mechanism of the singlet oxygen attack is still open to question although evidence indicates that reaction conditions, especially pH, may determine the primary products as well as the degradative pathway. Finally, we do not imply from this study that all of the so-called photodynamic oxidations of biological molecules occur via a singlet oxygen intermediate. If the sensitizer in an excited state is a very strong hydrogen atom abstractor then a free radical mechanism may result. However, the more efficient photodynamic dyes are not known for their hydrogen abstraction ability.

This work was supported by NASA grant NGR 39-009-008.

Received for publication 26 June 1969.

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