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The Photosensitized Reaction of Deoxyguanosine in the Presence of Methylene Blue*1

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The kinetics of the photosensitized decomposition of deoxyguanosine with methylene blue was studied by means of spectrophotometric measurements. The reaction rate increased with an increase in the concentration of the reactant or of oxygen up to a saturation level for each case at a given concentration of the dye. In a buffer solution of a lower phosphate concentration, a larger rate was observed. On the basis of these dependencies of the rate, the reaction scheme was discussed, and the ratios of some rate constants were estimated based on Schenck's mechanism. A preliminary NMR measurement of the protons of deoxyguanylic acid in D_2O suggested that the base-part of the nucleotide was specifically attacked by this photosensitized reaction.

Photodynamic action, namely, a dye-sensitized reaction of biological systems, is known in a wide variety of organisms.[1,2] For example, the muta-

*1 Presented at the 18th Annual Meeting of the Chemical Society of Japan (1965).

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1) J. D. Spikes and C. A. Ghiron, "Physical Processes in Radiation Biology," ed. by L. Augenstein, R. Mason and B. Rosenberg, Academic Press, New York (1964), p. 309.

2) J. D. Spikes and B. W. Glad, *Photochem. Photobiol.*, **3**, 471 (1964).

tions of bacteria and viruses are induced,[3,4] and the transforming DNA of *Diplococcus pneumoniae* is inactivated by the photodynamic action with methylene blue, acridine orange, *etc.*[5,6] Thus, deoxyribonucleic acid (DNA) seems to be a main target for this action in living organisms. In order to interpret the photosensitivity of organisms, knowledge about the relationship between the chemical alterations of DNA and the resultant biological phenomena is required. Studies of the dye-sensitized reaction of the components of nucleic acids would give basic information. Simon and van Vunakis[7-9] found that guanine derivatives in the components of DNA or RNA were preferentially destroyed by the sensitization of methylene blue. Each derivative consumes one mole of oxygen for each mole of substrate (reactant), accompanied by the loss of ultraviolet absorbance,[7] and the destructive reaction of deoxyguanosine is of the first-order.[8] They also studied the effects of dye concentration, light intensity, temperature, and pH on this reaction.[8]

In this article, we will discuss the reaction kinetics of the methylene blue-sensitized destruction of deoxyguanosine, with an emphasis on the effects of the concentrations of the substrate, oxygen, and the phosphate buffer on the reaction rate.

Experimental

Materials. Methylene blue was obtained from E. Merck Ag., while deoxyguanosine, deoxyadenosine, deoxycytidine, thymidine, deoxyguanylic acid, and 2-deoxy-D-ribose were from Sigma Chemical Co. All the reagents were used without purification. The molar extinction coefficient of methylene blue was measured to be 74000 at 665 mμ.

Irradiation. Methylene blue and the substrate were dissolved in a phosphate buffer. This mixed solution in silica cuvettes (1 cm in path length) was irradiated with visible light. Four photoflood lamps (500 W—100 V) were used at 75 V. Circulating-water filters (5 cm thick) were interposed between the lamps and the cuvettes to absorb thermic rays. The alignment for irradiation is shown in Fig. 1. The light intensity was adjusted so as to be the same at each cuvette. The reaction temperature was not controlled, since the rate constant of the destruction of deoxyguanosine did not vary between 6 and 58°C.[8]

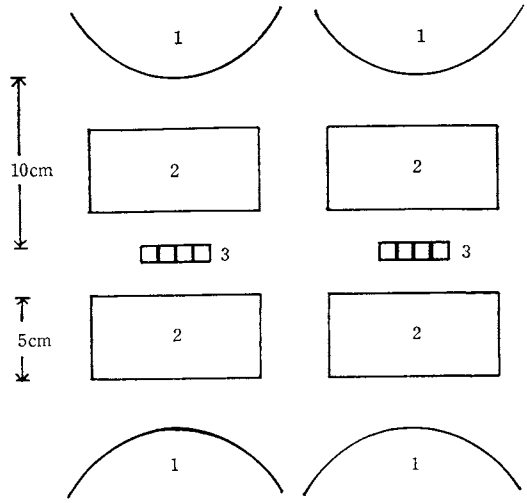

Fig. 1. Alignment of lamps (1), water-filters (2) and cuvettes (3).

Control of the Oxygen Concentration. Some cuvettes were designed to have provisions for bubbling mixtures of air and nitrogen gas in various ratios through sample solutions prior to and during irradiation. The concentration of oxygen in a solution was estimated from the solubility of gas at the experimental temperature. Nitrogen gas was passed through an alkaline-pyrogallol solution to eliminate the contaminating oxygen, and then through water, before being led into the reaction mixture.

Analysis. After irradiation, twenty mg of cation exchange resin (Dowex-50, Na-form) were added to the reaction mixture (3 m*l*) in order to remove methylene blue. The resin was added before the irradiation for the reference sample. The concentration of the substrate in the supernatant solution was measured with a Cary-14-spectrophotometer.

NMR Measurements. Deoxyguanylic acid and methylene blue were dissolved in D_2O to be 5% ($=1.7\times10^{-1}$M) and 3.3×10^{-4}M respectively. One milliliter of this mixed solution was poured into a cylindrical Pyrex reaction vessel (2.6 cm in diameter, 1.7 cm deep) with an inlet and an outlet tube. The thickness of the solution in this vessel was only 1.9 mm. After the inlet and the outlet had been sealed, the solution was irradiated using a photoflood lamp (500 W—100 V) working at 80 V at a distance of 8 cm over the vessel. The circulating water filter was also used in this case. The NMR spectra of the solution before and after the irradiation were measured with a Varian NMR spectrometer, HR-100. The relative signal intensity was determined by measuring the area of each signal on recording paper.

Results

Specificity of the Reaction. Our first interest was in ascertaining whether all the nucleosides constituting DNA submit to the sensitizing reaction of methylene blue. The specific destruction of deoxyguanosine in an aerated aqueous solution was confirmed by measuring the ultraviolet absorp-

3) R. W. Kaplan, *Nature*, **163**, 573 (1949).
4) H. Bohme and A. Wacker, *Biochem. Biophys. Res. Commun.*, **12**, 137 (1963).
5) J. S. Bellin and G. Oster, *Biochim. Biophys. Acta*, **42**, 533 (1960).
6) H. Fujita, E. Saito, K. Suzuki and A. Wada, *J. Rad. Res.*, **11**, (1970), in press.
7) M. I. Simon and H. van Vunakis, *J. Mol. Biol.*, **4**, 488 (1962).
8) M. I. Simon and H. van Vunakis, *Arch. Biochem. Biophys.*, **105**, 197 (1964).
9) M. I. Simon, L. Grossman and H. van Vunakis, *J. Mol. Biol.*, **12**, 50 (1965).

tion; this is in agreement with the finding of Simon and van Vunakis.[8] In connection with the specificity, the red shift of the visible absorption peak of the dye upon the addition of various nucleosides was measured (Fig. 2). The interaction of methylene blue with deoxyadenosine or deoxyguanosine is stronger than that with deoxycytidine or thymidine.

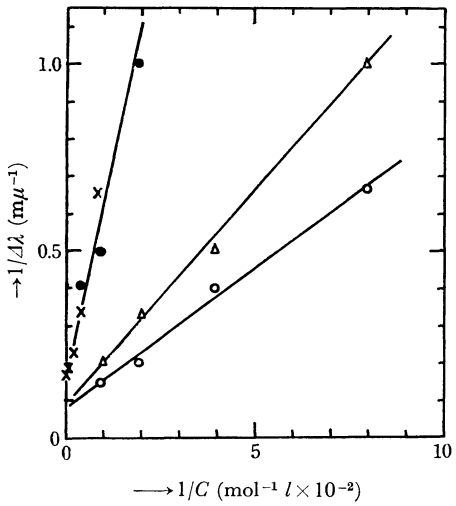

Fig. 2. Reciprocal plottings of extents of red shift ($\Delta\lambda$) and concentrations of nucleosides.
methylene blue, 5×10^{-6}M; phosphate, 1/15M. (pH 6.8); ×, thymidine; ●, deoxycytidine; △, deoxyguanosine; ○, deoxyadenosine.

The remaining concentration of the substrate decreased exponentially with an increase in the irradiation time up to 20 min, at least under the present conditions. Each reaction rate was estimated from the initial slope of the curve in the semi-logarithmic plotting. Deoxyguanylic acid was also decomposed by the irradiation of visible light in the presence of methylene blue when air was dissolved in the solution. Its reaction rate was found to be lower than that of deoxyguanosine (Fig. 3).

It was confirmed that the reaction rate of deoxyguanosine was independent on the pH between 5.4 and 6.6; this is consistent with the data of Simon and van Vunakis.[8] Thus, all the experiments below were performed with solutions in this pH range.

Effects of the Concentrations of Phosphate, the Substrate, and Oxygen. The reaction of deoxyguanosine was examined in 1×10^{-3} and 1×10^{-2}M phosphate buffers. The reaction rate increased with an increase in the concentration of the substrate up to a saturation level at a constant concentration of methylene blue (1×10^{-5}M), as may be seen in Fig. 3. In the solution with a lower phosphate concentration, a larger rate was observed. The phosphate anion may be connected with some

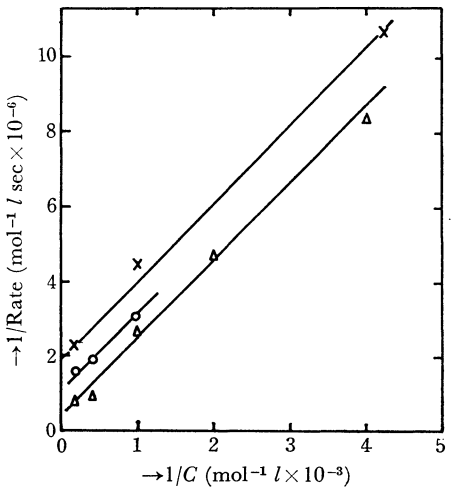

Fig. 3. Dependency of the reaction-rate on the concentration of substrate.
methylene blue, 1×10^{-5}M; air saturated; △ and ○, deoxyguanosine in 1×10^{-3} and 1×10^{-2}M phosphate buffer respectively; ×, deoxyguanylic acid in 1×10^{-2}M phosphate buffer.

irreversible reaction(s) of methylene blue in an aerated solution as well as in a deaerated solution.[10] The present finding may be interpreted as a result of competition between deoxyguanosine and phosphate.

It was also observed that the reaction proceeded with a larger rate with an increase in the concentration of oxygen up to a saturation level (Fig. 4). The reaction did not take place in a deaerated solution.

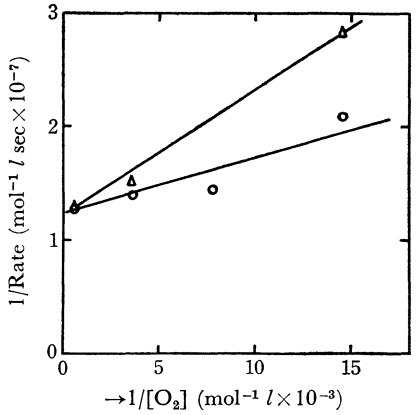

Fig. 4. Oxygen-effect on the reaction-rate.
methylene blue, 1×10^{-5}M; deoxyguanosine, 1×10^{-4}M; phosphate, 1×10^{-3}M (○) and 1×10^{-2}M (△).

Independency on Added 2-Deoxy-D-ribose. Varying amounts of 2-deoxy-D-ribose, which is

10) Y. Usui, H. Obata and M. Koizumi, This Bulletin, **34**, 1049 (1961), Y. Usui and M. Koizumi, *ibid.*, **34**, 1651 (1961).

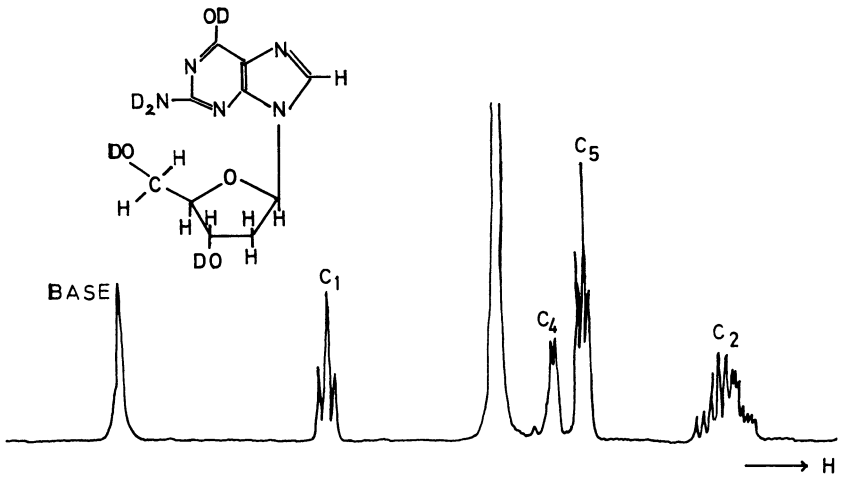

Fig. 5. NMR-Spectrum of deoxyguanylic acid in D_2O.

a component of deoxyguanosine, were added to a reaction mixture of deoxyguanosine (5×10^{-5}M) and methylene blue (1×10^{-5}M) in a phosphate buffer (1×10^{-3}M). Even if the additive was increased as high as 1×10^{-3}M, it however did not affect the photosensitized reaction.

NMR Measurements. As a preliminary experiment, the reaction of deoxyguanylic acid in D_2O was followed by NMR measurements. It did not have an adequate quantitative accuracy, but the decrease in the proton signal from the guanine base caused by the reaction was remarkable compared with that of the C_1-proton of the deoxyribose ring (See Fig. 5). The ratio between the signal intensity of the base and that of C_1 is plotted against the irradiation time in Fig. 6. Since methylene blue itself is also decomposed by such a long irradiation, this curve does not correspond to an actual dose-response of the reaction.

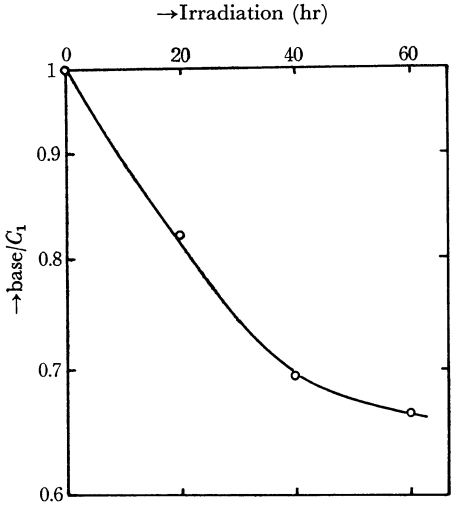

Fig. 6. Decrease of the ratio of NMR-signals, base to C_1, by the photosensitized reaction.
methylene blue, 3.3×10^{-4}M; deoxyguanylic acid, 5%.

Discussion

It was confirmed from spectral shifts that methylene blue interacts more strongly with purine nucleosides than with pyrimidine nucleosides. This is consistent with the results of Kodama *et al.*,[11] who observed that the spectral shifts of proflavine, acridine orange, and methylene blue are larger in the presence of apyrimidic acid than in the presence of apurinic acid, and who ascertained the base specificity of proflavine at the nucleoside level as well as at the polymer level on the basis of a measurement of the difference spectra. The specific destruction of deoxyguanosine, however, can not be accounted for by the specific interaction of methylene blue with deoxyguanosine, since deoxyadenosine was not destroyed by the present reaction and since the photosensitized reaction of deoxyguanosine was carried out at such a low concentration that the red shift was not observed.

Oster *et al.*[12] reported the photo-oxidation of 2,5-diaminotoluene using proflavine as a sensitizer, and postulated that the long-lived, electronically-excited (probably triplet) state of the dye reacted with oxygen to form some dye peroxide, which in turn oxidized the substrate. The reaction scheme for the present system can be built up in the same way:

$$D + h\nu \rightarrow D^* \quad (1)$$

$$D^* \rightarrow D + h\nu_f \quad (2)$$

$$D^* \rightarrow D' \quad (3)$$

11) M. Kodama, Y. Tagashira and C. Nagata, *J. Biochemistry.*, **64**, 167 (1968).

12) G. Oster, J. S. Bellin, R. W. Kimball and M. E. Schrader, *J. Amer. Chem. Soc.*, **81**, 5095 (1959).

$$D' \rightarrow D + h\nu_p \quad (4)$$

$$D' + D \rightarrow 2D \quad (5)$$

$$D' + P \rightarrow \text{product} \quad (5')$$

$$D' + O_2 \rightarrow DO_2 \quad (6)$$

$$DO_2 \rightarrow D + O_2 \quad (7)$$

$$DO_2 + D \rightarrow 2D + O_2 \quad (8)$$

$$DO_2 + P \rightarrow D + \text{product} \quad (8')$$

$$DO_2 + S \rightarrow D + SO_2 \quad (9)$$

where D is the ground state of the dye; D*, the singlet excited state of the dye; D′, the triplet excited state of the dye; P, phosphate; S, the substrate; $h\nu_f$, fluorescence and $h\nu_p$, phosphorescence. If the steady-state treatment is applied to each intermediate, namely, D*, D′, and DO_2, the rate formula may be expressed as follows;

$$-d[S]/dt = k_1 \cdot [D] \Big/ \left[\left(1 + \frac{k_7}{k_9 \cdot [S]} + \frac{k_8 \cdot [D] + k_{8'} \cdot [P]}{k_9 \cdot [S]} \right) \times \left(1 + \frac{k_4}{k_6 \cdot [O_2]} + \frac{k_5 \cdot [D] + k_{5'} \cdot [P]}{k_6 \cdot [O_2]} \right) \cdot \left(1 + \frac{k_2}{k_3} \right) \right]$$

where each k is the rate constant at the elementary process expressed by the subscript. This means that the plot of 1/rate *vs.* 1/[S] should be a straight line when [D], [O_2] and [P] are constant, while the plot of 1/rate *vs.* 1/[O_2] should be linear when [D], [S] and [P] are constant. The reciprocal plot of the experimental values approximately satisfied the above scheme.

Since the quantum yields of the fluorescence and the phosphorescence of methylene blue might be rather small, it may be postulated that $k_2 \ll k_3$ and $k_4 \ll k_6 \cdot [O_2]$. Furthermore, since the slope in the reciprocal plotting on the effect of the substrate concentration at $[P] = 1 \times 10^{-2}$M was nearly the same as that at $[P] = 1 \times 10^{-3}$M, the $k_{8'} \cdot [P] \ll k_7 + k_8 \cdot [D]$ relation is plausible. Thus, the following ratios of the rate constants could be roughly estimated. Comparing the above formula with the results on the effect of the oxygen concentration at different concentration of phosphate ions, we obtained $k_5/k_6 \approx 2.5$ and $k_5/k_{5'} \approx 4 \times 10^2$; that is, the difference between the probabilities of the deactivation of long-lived excited dye molecules (D′) by unexcited ones and of the reaction of D′ with molecular oxygen is not large, and the probability of reaction 5′ is very small. From the dependency on the substrate concentration at two concentrations of the dye, *i.e.*, 1×10^{-5} and 5×10^{-6}M, $k_7/k_8 \approx 2 \times 10^{-5}$M, $k_8/k_9 \approx 3 \times 10$, and $k_7/k_9 \approx 5 \times 10^{-4}$M.

In the reaction mechanism described above, the conception of the molecular complex (DO_2) was accepted as an intermediate on the basis of the reaction scheme proposed by Schenck.[13] However, even if one considers that the singlet excited state of the oxygen molecule, $O_2(^1\Delta_g)$[14,15] or $O_2(^1\Sigma_g)$,[15] is produced by process (6), the final formula for the kinetics is the same.

The results of the NMR measurements were not incompatible with the study of the kinetics. It may be concluded that, in the reaction of deoxyguanosine or deoxyguanylic acid with methylene blue, the guanine residue is preferentially subjected to the reaction, whereas the deoxyribose residue is rather stable.

According to Waskell *et al.*,[16] guanosine was decomposed by the sensitization of methylene blue to ribosyl urea, free ribose, guanidine, and traces of urea in the earlier stage of irradiation, while the ribose moiety disappeared in the latter stage. The preferential destruction the base component suggests that the secondary reaction of such intermediates as radicals, which may be formed in the primary process, with the sugar component is not appreciable.

The reaction kinetics of deoxyguanosine was studied as a sole solution in the present case. If its reaction is followed in the presence of the three other nucleosides, information about the reaction mechanism as a state closer to the actual system can be obtained. Furthermore, if a competitive reaction between DNA and deoxyguanosine is studied, the $k_{DNA}/k_{nucleoside}$ ratio must be estimated. The biological significance of the selective destruction of the guanine derivatives in DNA or RNA will be thus confirmed.

13) G. O. Schenck and E. Koch, *Z. Elektrochem.*, **64**, 170 (1960).

14) C. S. Foote and S. Wexler, *J. Amer. Chem. Soc.*, **86**, 3880 (1964).

15) D. D. Morgan and M. Orchin, Abstract for The 5th International Congress on Photobiology, New Hampshire, U.S.A. (1968), p. 30.

16) L. A. Waskell, K. S. Sastry and M. P. Gordon, *Biochim. Biophys. Acta*, **129**, 49 (1966).