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Oxidation Photosensitized by Tetracyclines

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Abstract □ Irradiation with 365-nm UV light of aerated aqueous solutions of tetracycline gives rise to oxygen uptake when the pH of the solution is above 7.5. The kinetics of the reaction were followed using a polarographic oxygen electrode at a range of pH values for seven currently prescribed tetracyclines. Variation of tetracycline concentration, UV light intensity, and temperature showed the characteristics normally associated with a sensitized photo-oxygenation mechanism rather than a free-radical process. Copper(II) ions inhibited the photo-oxidation of tetracycline, apparently by complex formation. The tetracyclines were tested for photosensitizing capability with oxidizable acceptors. In aqueous solution, no photosensitizing effect could be seen, but methanol solutions of 2,5-dimethylfuran and *dl*-limonene were oxidized at considerably increased rates when small amounts of tetracyclines were present. This observation has implications for the mechanism of *in vivo* photosensitivity reactions that occur when tetracyclines are taken internally.

Keyphrases □ Tetracyclines, various—photosensitized oxidation, reaction kinetics followed using polarographic oxygen electrode, effect of pH and copper(II) ions □ Photosensitized oxidation—various tetracyclines, reaction kinetics followed using polarographic oxygen electrode, effect of pH and copper(II) ions □ Oxidation, photosensitized—various tetracyclines, reaction kinetics followed using polarographic oxygen electrode, effect of pH and copper(II) ions □ Polarography—determination, rate of oxygen uptake by solutions of various tetracyclines irradiated with UV light, effect of pH and copper(II) ions □ Antibacterial agents—various tetracyclines, photosensitized oxidation, reaction kinetics followed using polarographic oxygen electrode, effect of pH and copper(II) ions

Photosensitivity reactions have been reported for the tetracyclines after exposure of the patient to strong sunlight (1). The phenomenon of "photodynamic action" has been recognized since 1900, when it was discovered that microorganisms can be killed when exposed to light in the presence of oxygen and sensitizing dyes (2). The basic mechanism is the photosensitized oxidation of the adsorbate or substrate by molecular oxygen (3, 4). All tetracycline antibiotics have a strong absorption in the near UV region at about 365 nm, the first requirement for a sensitizer of photodynamic action.

Little information is available, however, concerning the reactions of tetracyclines following absorption of UV light. Chlortetracycline, tetracycline, and oxytetracycline lost significant antibiotic potency when irradiated with visible light in solution with riboflavin, which probably acted as a photosensitizer (5). Leeson and Weidenheimer (6) reinvestigated this system at pH 4.5 and concluded that the loss of tetracycline activity could be suppressed by the addition of ascorbic acid. Thus, an oxidative pathway was implied for degradation of tetracycline after irradiation.

This paper reports a more detailed study of the photooxidation of seven currently prescribed tetracyclines, together with some experiments that indicate that the tetracyclines can act as photosensitizers for the oxidation of suitable acceptor molecules.

EXPERIMENTAL

Samples of tetracycline[1] (I), oxytetracycline[1], doxycycline[1], metha-

[1] Pfizer Laboratories.

Table I—Initial Rate of Oxygen Uptake by Irradiated Tetracycline Solutions at pH 9.0 and 30°

[Tetracycline], $M \times 10^4$	Initial Rate[a], moles/liter/min $\times 10^6$	Transmittance[b] at 373 nm, %
0.5	1.7 ± 0.2	17.8
1.0	3.5 ± 0.1	3.2
1.5	5.3 ± 0.3	<0.01
3.0	5.8 ± 0.2	<0.01
5.0	5.8 ± 0.2	<0.01
6.0	5.7 ± 0.3	<0.01
10.0	5.4 ± 0.2	<0.01

[a] Mean of at least three determinations from initial slope of P_{O_2} recorder trace. [b] Calculated for 1-cm path length and molar absorptivity of $1.5 \times 10^4 M^{-1}$ cm^{-1} at pH 9.0.

cycline[1], chlortetracycline[2], demeclocycline[2], and minocycline[2] of the purest grade available commercially were used without further purification. The absorbance ratio method of McCormick *et al.* (7) showed that the purity of tetracycline, oxytetracycline, and chlortetracycline was at least 99%.

Rates of oxygen uptake by solutions irradiated by UV light were determined using the polarographic oxygen electrode apparatus described previously (8). In the procedure used, 2 ml of freshly prepared solution of I in buffer was mixed with 200 ml of air-saturated buffer solution before filling the reaction vessel. Buffers (0.05 *M*) were prepared in double-distilled water using analytical grade materials as follows: pH 4.0–4.9, acetate; pH 6.0–8.4, phosphate; pH 9.0, tromethamine; and pH 10.0–10.8, carbonate.

For experiments in which the tetracyclines were tested as photosensitizers, benzyl alcohol[3], benzaldehyde[3], *dl*-limonene[4], and 2,5-dimethylfuran[5] were purified by distilling twice under high vacuum in an all-glass apparatus at room temperature. Sodium xanthine[6], α-tocopherol[6], *dl*-tyrosine[6], and *l*-phenylalanine[6] were used as received. Limonene, dimethylfuran, and α-tocopherol are almost insoluble in aqueous systems, so experiments with these compounds were performed in methanol solution.

The rate data were recorded with the oxygen electrode operating successfully in methanol, although it was designed for operation in aqueous phases only (9). The validity of oxygen uptake data measured in methanol solution was verified by experiments with benzaldehyde for which the mechanism is known (8). Moreover, the stability of the oxygen electrode and the reproducibility of readings were greatly improved in methanol solutions; this finding may be attributed to the greater concentration of oxygen in air-saturated methanol ($2.06 \times 10^{-3} M$) compared to air-saturated water ($2.35 \times 10^{-4} M$) at 30° (10).

RESULTS

Preliminary Measurements with Tetracycline—The rate of oxygen uptake by UV-irradiated I in solution was examined over pH 4.0–10.8. No significant oxygen uptake occurred below pH 8, although there were changes in absorbance[7] at pH 4 and 5 in the 240–280-nm range, corresponding to epimerization (11). The loss of antibiotic activity of irradiated I reported (6) at pH 4.5 is thus largely due to epimerization, which occurs only between pH 2 and 6 (11).

During irradiation at pH 9.0, oxygen consumption was accompanied by color change of the solution from yellow to pink, red, or brown (depending on the extent of oxidation), with the appearance of a broad absorption centered at 530 nm. The absorbance decreased at 373 nm, which is the λ_{max} for I at pH 9.0, and disappeared completely if irradiation was continued. There was no absorption in the 400–450-nm region, indicating that neither acid degradation product (12) (anhydrotetracycline or epianhydrotetracycline) was formed during photo-oxidation. The oxygen uptake from irradiated I solutions continued (at a much reduced rate) after the lamp had been switched off, indicating that the product(s) of I photo-oxidation are labile. Analysis of these solutions with iodine immediately after irradiation produced no evidence of peroxides.

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[3] British Drug Houses, Poole, England.
[4] Eastman Kodak, Rochester, N.Y.
[5] Fluka AG, Switzerland.
[6] Nutritional Biochemicals Corp., Cleveland, Ohio.
[7] Varian Techtron model 635 UV-visible spectrophotometer.

Table II—Effect of Edetate Disodium and Copper(II) Ions on Tetracycline Photo-Oxidation at pH 9.0 and 30°[a]

[Edetate Disodium], $M \times 10^5$	[Cu^{2+}], $M \times 10^5$	Initial Rate, moles of Oxygen/liter/min $\times 10^6$
0	0	3.5 ± 0.1
10	0	3.5 ± 0.2
0	1.0	2.2 ± 0.1
0	2.5	1.46 ± 0.10
0	5.0	1.17 ± 0.10
0	40	0.1 ± 0.1

[a] [Tetracycline] = $10^{-4} M$ in all experiments.

Correlation of the amount of oxygen consumed with the depletion of I, as indicated by the spectral change at 373 nm, established that 1.06 ± 0.08 moles of oxygen reacted per mole of I. These experiments were performed at two concentrations of I; to keep secondary reactions to a minimum, irradiation was continued until no more than 15% of the dissolved oxygen was consumed.

To test for general acid or base catalysis by the buffer components, a characteristic of the epimerization reaction (7), the irradiation of I was performed at pH 9.0 in: (*a*) 0.05 *M* tromethamine buffer, (*b*) 0.05 *M* carbonate buffer, and (*c*) unbuffered solution adjusted to pH 9.0 with 1 *M* NaOH solution. After 20 min of irradiation, the pH of the unbuffered solution had fallen to 8.6 but the initial rate of oxygen uptake was the same for all three solutions, indicating that there is no participation by the buffers in the reaction nor any effect of ionic strength.

Temperature variation over 25–40° showed that the reaction rate was independent of temperature. The incident light intensity was varied as previously described (8), and the reaction rate was directly dependent. Both these observations are characteristic of a primary photochemical process rather than a free radical chain mechanism (13).

Variation of Tetracycline Concentration—Table I shows the measured initial rate of oxygen uptake as the I concentration was varied from 5×10^{-5} to $10^{-3} M$. Straight-line (*i.e.*, zero-order) recorder traces of oxygen pressure (P_{o_2}) *versus* time were obtained for $[I] \geq 10^{-4} M$. Below $10^{-4} M$, the traces were curved, but straight lines were obtained for plots of log P_{o_2} *versus* time. The solubility of oxygen in dilute buffer is $2.35 \times 10^{-4} M$ (10), so I concentration was rate limiting here and the rate can be said to be first order in I concentration. At the lower I concentrations, the rate was proportional to [I]; above $3 \times 10^{-4} M$, the light intensity was the limiting factor, as indicated by the percent transmittance values given in Table I. This effect also was observed previously (5, 6).

Effect of Edetate Disodium and Copper on Tetracycline Photo-Oxidation—It is well known that traces of metal ions catalyze radical chain oxidation (14) and that I complexes with many metal ions (15). The experiments summarized in Table II showed that the addition of edetate disodium to the buffer had no effect on the photo-oxidation rate, indicating that there are no accelerating effects of trace metal ions. The addition of copper(II) ions, however, led to a decrease in rate, indicating that complexed I is not susceptible to photo-oxidation. The fact that a complex was formed was indicated by a shift in the absorption maximum of I from 373 to 405 nm. The absorbance of the complex at 365 nm (the wavelength of maximum output of the UV lamp) remained sufficient to initiate photo-oxidation, provided that the complex was susceptible.

Comparative Study of Seven Tetracyclines—The initial rates of photo-oxidation for seven tetracyclines at a range of pH values are recorded in Table III. Minocycline showed little reactivity under the present conditions; methacycline also reacted more slowly than the other five tetracyclines. In general, the reaction rate was at a minimum at neutral pH and increased as the alkalinity of the solution was raised. Doxycycline showed a maximum oxidation rate at pH 9.0, while tetracycline exhibited a similar maximum at pH 10.0.

The UV spectral changes described earlier for tetracycline were observed for the other members of the group, the extent of the change in the 360–380-nm peak being directly proportional to the oxygen uptake. Chlortetracycline alone had a significant dark reaction, not involving oxygen uptake but involving a 10% reduction in absorbance of the 380-nm peak at pH 9.0 in 1 hr, the time course of the photo-oxidation reaction. Hughes and Wilson (16) showed that isochlortetracycline forms upon heating alkaline solutions of chlortetracycline while other tetracyclines are less labile in this respect.

Tetracyclines as Photosensitizers—The fact that I is the primary UV-absorbing species for its own photo-oxidation suggests that it may

Table III—Initial Rates of Photo-Oxidation of Tetracyclines at 30° and Various pH Values

Compound	Concentration, $\times 10^4 M$	Initial Rate[a] of Oxygen Uptake (moles/liter/min $\times 10^6$) at pH 6.0	pH 7.0	pH 8.0	pH 9.0	pH 10.0	pH 10.8
Tetracycline	0.5	0	0.23	0.82	1.72	2.08	1.67
	2.0	0	0.24	1.40	5.3	6.2	4.4
Oxytetracycline	0.5	0	0.10	0.60	1.44	1.76	—
	2.0	0	0.24	1.35	3.1	4.2	—
Chlortetracycline	0.5	0	0.32	1.02	1.70	1.84	—
	2.0	0	0.89	1.6	3.3	4.2	—
Demeclocycline	0.5	0	0.22	0.8	1.33	2.22	—
	2.0	0	0.55	1.3	2.32	4.1	—
Doxycycline	0.5	0	0.66	1.4	1.68	1.12	—
	2.5	0	0.82	3.1	4.3	1.96	—
Methacycline	0.5	0	0.20	0.32	0.51	0.82	—
	2.0	0	0.32	0.73	1.20	1.32	—
Minocycline	0.5	0	<0.1	0.14	0.20	0.30	—
	2.0	0	<0.1	0.18	0.32	0.40	—

[a] Mean of three determinations from initial slope of P_{O_2} recorder trace.

be capable of sensitizing the photo-oxidation of other molecules. Various concentrations (10^{-5}–10^{-4} M) of I were tested for sensitizing capability with the following systems:

System 1—Benzyl alcohol or benzaldehyde (2×10^{-2} M) in aqueous solution at pH 4.0 and 9.0, as representative of compounds susceptible to free radical oxidation (8).

System 2—Xanthine, phenylalanine, or tyrosine (10^{-2} M) in aqueous solutions at pH 6.0 and 8.0. Both purines (17) and aromatic amino acids (18) undergo sensitized photo-oxidation in alkaline solution.

System 3—α-Tocopherol (2×10^{-2} M), a natural antioxidant that undergoes both free radical autoxidation (19) and dye-sensitized photo-oxidation (20) in methanol solution.

System 4—2,5-Dimethylfuran or *dl*-limonene in methanol solution. These agents were used (21, 22) previously as model compounds in dye-sensitized photo-oxygenations and chemical oxygenations to test for the participation of singlet molecular oxygen.

For Systems 1–3, the oxygen uptake upon irradiation was very slight and corresponded to that recorded in control experiments with I or substrate alone. Dimethylfuran and limonene, however, were oxidized significantly in the presence of all tetracyclines except minocycline (Table IV). A 10-fold change in [I] produced less than double the rate of limonene oxidation, while the doubling of doxycycline increased the rate of dimethylfuran oxidation by only 6%. Also, the absorbance of the reaction mixtures at 370 nm decreased by only about 2%, and the tetracyclines by themselves in methanol did not absorb measurable amounts of oxygen in the time course of the irradiation (1 hr). These observations establish the role of the tetracyclines as photosensitizers.

Table IV—Photo-Oxidation of Limonene and 2,5-Dimethylfuran in Methanol Solution Sensitized by Tetracyclines at 30°

Sensitizer	Concentration $\times 10^4 M$	Substrate Concentration, M	Rate of Oxygen Uptake, moles/liter/min $\times 10^6$
	Limonene		
None	—	0.062	0.2
Tetracycline	0.1	0.062	1.8
Tetracycline	1.0	0.062	3.2
Oxytetracycline	1.0	0.062	2.1
Doxycycline	1.0	0.062	2.4
Chlortetracycline	1.0	0.062	3.8
Demeclocycline	1.0	0.062	3.7
Methacycline	1.0	0.062	2.6
Minocycline	1.0	0.062	0.5
	2,5-Dimethylfuran		
None	—	0.038	<0.2
Tetracycline	0.5	0.038	3.4
Tetracycline	0.5	0.055	4.2
Tetracycline	0.5	0.104	7.5
Oxytetracycline	0.5	0.055	2.7
Doxycycline	0.5	0.055	2.0
Doxycycline	1.0	0.055	2.2
Chlortetracycline	0.5	0.055	3.5
Demeclocycline	0.5	0.055	3.6
Methacycline	0.5	0.055	2.8
Minocycline	0.5	0.055	1.2

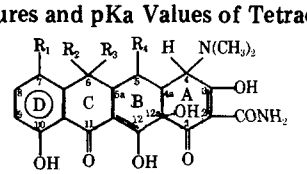
DISCUSSION

The major kinetic characteristics observed for the photo-oxidation of I in aqueous alkaline solution may be summarized as follows: (*a*) dependence on [I] only at low concentrations, (*b*) direct dependence on incident light intensity, (*c*) independence of temperature, and (*d*) use of oxygen in a 1:1 stoichiometry with I. These are the characteristics of a photosensitized oxygen addition or photo-oxygenation process (2) rather than of a free radical chain mechanism such as occurs with benzaldehyde (8).

The structures of the tetracyclines in this study are given in Table V together with the known pKa values. While it is difficult to correlate differences in structure with the differences in photo-oxidative reactivity, the rate variation with pH is clearly related to the state of ionization of the tetracyclines in mildly alkaline solution and is similar to the pH profile observed for the photo-oxidation of several aromatic amino acids (18). The pKa values are attributed (23) respectively to the tricarbonyl system of ring A (pK_1), the dimethylammonium function of ring A (pK_2), and the phenolic β-diketone system in the C-10–11–12 region (pK_3). From the pKa values, I is said to exist as a zwitterion at neutral pH and increasingly as the monoanion as the pH is raised above 8. On this basis, the monoanion would appear to be the reactive species.

The photo-oxidation reaction is accompanied by a loss of the absorption peak at about 370 nm, which is due to the chromophore grouping in the BCD ring (7, 24) containing the ionizable system corresponding to pK_3. The dissociation of this group is essentially unaffected by the 7-chloro substitution, but it is not known whether the different substituents

Table V—Structures and pKa Values of Tetracyclines



Name	R_1	R_2	R_3	R_4	pK_1	pK_2	pK_3	Reference
Tetracycline	H	CH_3	OH	H	3.30	7.68	9.69	23
Chlortetracycline	Cl	CH_3	OH	H	3.30	7.44	9.27	23
Oxytetracycline	H	CH_3	OH	OH	3.27	7.32	9.11	23
Doxycycline	H	CH_3	H	OH	3.4	7.7	9.7	30
Demeclocycline	Cl	H	OH	H	3.30	7.16	9.25	31
Methacycline	H	—CH_2—		OH	—	—	—	—
Minocycline	$N(CH_3)_2$	H	H	H	—	—	—	—

in methacycline and minocycline cause a significant alteration in the pK value and, therefore, the concentration of the monoanion. There is no marked difference in the absorption peak at 370 nm for all tetracyclines studied here.

The addition of copper(II) ions shifts the 370-nm absorption to longer wavelength and renders the complex stable to photo-oxidation at pH 9.0. This tends to implicate the C-10–11–12 grouping as the binding site for metal ions. Mitscher *et al.* (24) concluded, from optical rotatory dispersion studies, that chelation occurred first in the BCD ring and then in the A ring as the pH was raised above 10. Williamson and Everett (25) recently suggested, on the basis of NMR studies in dimethyl sulfoxide, that the tricarbonylmethane function of ring A is the chelating group, but the solvent difference may give rise to a different binding behavior.

The decreased rate of photo-oxidation above pH 10.0 for I and pH 9.0 for doxycycline are not easily attributable to the decrease in the concentration of the monoanion in solution; if the monoanion is the only oxidizable species of I, the maximum rate should occur at pH 8–9, where its concentration would be at a maximum on the basis of the pK values.

The demonstration that the tetracyclines are capable of acting as photosensitizers for the oxygenation of suitable acceptor molecules has relevance to the photosensitivity reactions observed following the ingestion of I and its analogs. Dimethylfuran and limonene are acceptors (A) of energy transferred from $^1O_2$, the excited singlet molecular oxygen (21). This implies that excited I is capable of interacting with $^3O_2$ (ground-state triplet oxygen) to give singlet oxygen according to Scheme I.

$$(I) \xrightarrow{h\nu} (I)^*$$

$$(I)^* + {}^3O_2({}^3\textstyle\sum_g^-) \rightarrow (I) + {}^1O_2({}^1\Delta_g)$$

$$^1O_2 + A \rightarrow AO_2$$

Scheme I

This phenomenon has been principally studied using dyes that absorb in the visible region, such as methylene blue or rose bengal, since the energy difference between the triplet oxygen ground state and the singlet excited state is only 92 kJ (22 kcal), which corresponds to a wavelength of 1270 nm (21). It also usually requires that the dye pass to the excited triplet state, which has a longer lifetime in which to effect the energy transfer.

Foote (26) stated that if the oxidation proceeds purely through singlet oxygen as an intermediate, it should produce the same rate for all acceptors at constant sensitizer and oxygen concentrations. Thus, the similarity in rates observed with limonene and dimethylfuran is in agreement with this concept. No photosensitizing capability of I could be demonstrated in aqueous solution, so it is clear that the acceptor or substrate molecule and solvent medium have to be specifically matched to the sensitizer for greatest efficiency. Young *et al.* (27) suggested that the lifetime of singlet oxygen in photosensitized oxidations increases as the polarity decreases. This aspect could not be tested, since the amino acids and purines are insufficiently soluble in methanol and limonene and dimethylfuran are insoluble in water.

Reports of *in vivo* photosensitivity have implicated demeclocycline more frequently than the other members of the group (28, 29). The results in Table IV indicate some differences in photosensitizing efficiency, with demeclocycline, chlortetracycline, and tetracycline being the most efficient. The relative inactivity of minocycline is due to a shift of the λ_{max} in methanol solution to 345 nm, so that its molar absorptivity at 365 nm is approximately half that of the other tetracyclines. The investigation is continuing with systems that can be related more closely to the skin.

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