Photochemical Oxidation of Tetracycline in Aqueous Solution

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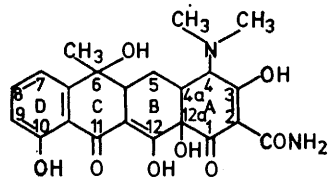
Irradiation of an aqueous solution of tetracycline at pH 9 with u.v. light from a medium pressure mercury lamp (filtered through Pyrex glass to give wavelengths >290 nm) produces a red product with $\lambda_{max.}$ 534 nm. The red product is only produced in the presence of oxygen, one mole of oxygen being absorbed per mole of tetracycline photolysed. When the photolysis is carried out at *ca.* 3 °C an intermediate compound is produced ($\lambda_{max.}$ 357 nm). On warming this irradiated solution the red product is formed in a dark reaction for which the activation energy is 90 kJ mol^{-1}. The red product has a pK_a value of 7.6, the protonated form being orange-yellow ($\lambda_{max.}$ 442 nm). The red product is reduced by sodium dithionite solution to a leuco form which is reoxidised to the original compound by oxygen of the air. The red product is also reduced photochemically; flash photolysis reveals the intermediacy of a semiquinone radical in this process. These properties are typical of a quinone ring system. The spectral and chemical properties of the red product are consistent with its being 4a,12a-anhydro-4-oxo-4-dedimethylaminotetracycline. The mechanism of formation of the red product involves photodeamination followed by reaction of the tetracycline radical with molecular oxygen to form a peroxyl radical, which abstracts a hydrogen atom to give a hydroperoxide. Decomposition of the hydroperoxide by loss of water yields an oxo group at position 4. In the final dark reaction, water is lost across the C(4a)–C(12a) bond to form the red quinone.

THE photochemistry of tetracycline antibiotics is of interest because of the incidence of phototoxicity in patients being treated with these drugs.[1] In order to ascertain the possible causes of this photosensitisation we have investigated recently the photochemistry of certain members of this class of antibiotic in aqueous solution. It was during the course of this investigation that we noticed the formation of a red compound when



(I)

aerated aqueous solutions of tetracycline (I) were irradiated with light of wavelength >290 nm. This observation was recently independently reported by Wiebe and Moore.[2] These workers showed that during the formation of the red product one mole of oxygen is absorbed per mole of tetracycline photolysed. They also showed that the rate of photo-oxidation of tetracycline is directly proportional to light intensity and is independent of temperature in the range 25—40 °C. The nature of the red product and the mechanism of its formation is the subject of our paper.

EXPERIMENTAL

Oxygen absorption by irradiated solutions of tetracycline was measured in a constant pressure apparatus based on the design of Bolland and Cooper.[3] The irradiation vessel had an 'oxygenation stirrer' which ensured saturation of the solution with oxygen throughout the irradiation.[4] The light source was a Hanovia 100 W medium pressure mercury vapour lamp. Solutions were irradiated through quartz or alternatively Pyrex glass, the latter being used when it was desired to cut out u.v. light of wavelengths <290 nm.

For observation of spectral changes, solutions of tetracycline were irradiated in a circular quartz cell (20 mm i.d., 10 mm path length). The cell, which had a narrow inlet tube fitted with a B7 stopper, was placed in a constant temperature bath at 25 ± 0.1 °C. A quartz window in the side of the bath permitted light from a 100 W medium pressure mercury lamp to enter the solution. A Pyrex glass filter was placed in front of the cell in most experiments to cut out u.v. light of wavelengths <290 nm. After suitable periods of irradiation, accurately controlled by a shutter, the u.v.–visible absorption spectrum of the solution was measured in a Pye–Unicam SP 1800 spectrophotometer.

For irradiation at *ca.* 3 °C the tetracycline solution was placed in a quartz tube which was contained in a Pyrex beaker full of ice–water. The temperature of the bath was maintained at 0 °C throughout the irradiation by adding ice to the beaker from time to time. The quartz tube was 12 cm from an Hanovia medium pressure mercury lamp. A 3 ml portion of the irradiated solution was withdrawn periodically and transferred to a 10 mm path-length cell for spectrophotometric measurement.

The flash-photolysis apparatus has been described.[5] Solutions were flashed in 15 mm i.d., 200 mm path-length cells; both quartz and Pyrex cells were used. Prior to flash photolysis, solutions were bubbled with nitrogen or oxygen as required.

The apparatus used to determine hydrogen peroxide consisted of a combined oxygen electrode and Perspex cell in which the solution was stirred magnetically (Rank Brothers, Cambridge). The output of the electrode was displayed on a Servoscribe linear recorder. A 3 ml sample of the photolysed solution was placed in the cell and the stirrer was started. When a steady chart trace had been obtained, 50 μl of a solution of catalase was added by means of a syringe. The amount of oxygen liberated (and hence the concentration of hydrogen peroxide in the photolysed solution) was determined from the recorder trace.

Preparation of the red product for further study was achieved as follows. A saturated solution of tetracycline hydrochloride in 0.1M-disodium hydrogenphosphate (150 ml) was placed in a Pyrex water-cooled vessel and oxygen was bubbled through the solution for 30 min. The solution was irradiated for 60 min at a distance of 30 cm from an Hanovia 500 W medium pressure mercury lamp. Throughout the irradiation, bubbling with oxygen was continued. The resulting red solution was concentrated, by rotary

evaporation at 40 °C, to 20 ml. Butan-1-ol (100 ml) was added and the mixture was shaken. The butan-1-ol layer was discarded. This process, which was repeated several times, efficiently removed unchanged tetracycline and resulted in the crystallisation of the red product together with disodium hydrogenphosphate. After washing three times *with* butan-1-ol, the material was dried under vacuum in darkness. Because disodium hydrogenphosphate appeared to stabilise the red product it was not separated from it. Solutions of the red product containing disodium hydrogenphosphate were made up as required and adjusted to a suitable pH and absorbance (at 534 nm) for further investigation.

Materials.—Tetracycline hydrochloride was a generous gift from the Lederle Laboratories. Disodium hydrogenphosphate was AnalaR grade. Nitrogen was B.O.C. ' white spot ' grade. Distilled water was used in the preparation of all solutions.

RESULTS

Figure 1 shows the absorbance changes that occurred when an air-saturated solution of tetracycline, in 0.1M-disodium hydrogenphosphate at pH 9.0, was irradiated in the circular cell. There was a fall in the absorbance of the longest wavelength band ($\lambda_{max.}$ 375 nm) and a corresponding growth in an absorption band, having $\lambda_{max.}$ 534 nm, which is responsible for the colour of the red product. An isosbestic point at *ca.* 412 nm was observed in the superimposed absorption spectra obtained at various times during the irradiation. This indicates a simple conversion of tetracycline to the red product.

The red product was not formed when the solution of tetracycline was bubbled with nitrogen for 15 min prior to irradiation.

When an air-saturated solution of tetracycline at pH 9.0 was irradiated through quartz glass instead of Pyrex the fall in absorbance at 375 nm was more rapid than that observed using Pyrex glass but the red product did not appear in any quantity. We also found that if, after producing the red product by photolysis through a Pyrex filter, photolysis was continued after removal of this filter, the red product itself was photolysed. It appears therefore, than when tetracycline is irradiated through quartz, the red product is photolysed almost as rapidly as it is produced, and therefore does not accumulate in the irradiation vessel.

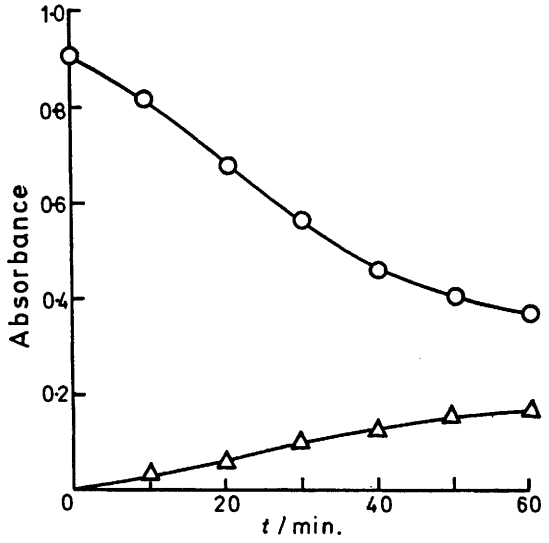


FIGURE 1 Decrease in absorbance at 375 nm (○) and simultaneous increase in absorbance at 534 nm (△) during the photolysis (through Pyrex) of tetracycline in air-saturated 0.1M-Na_2HPO_4 solution (pH 9.0) to form the red product

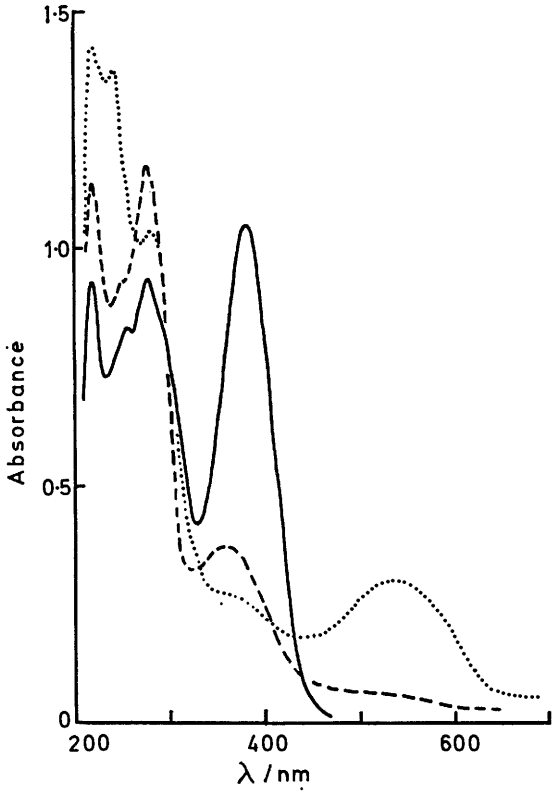


FIGURE 2 Absorption spectra of tetracycline in 0.1M-Na_2HPO_4 solution at pH 9.0, before photolysis (——); after photolysis through Pyrex at 3 °C (– – – –); red product formed after warming the irradiated solution (· · · ·)

When a solution of tetracycline in 0.1M-disodium hydrogen-phosphate solution (pH 9.0) was irradiated at *ca.* 3 °C for 1 h, the red product was not formed but there was a marked fall in the absorbance at 375 nm and an increase at *ca.* 213 and 270 nm. The absorption spectrum of the product showed a band having $\lambda_{max.}$ 357 nm and a very weak absorption in the visible region which gave the irradiated solution a slight brown colour. The photolysed solution gave a negative starch–iodide test. On equilibrating a 4 ml portion of the irradiated solution to 25 °C, the red product was gradually produced, and there was a corresponding fall in absorbance at 357 nm. The spectral changes are shown in Figure 2.

The rate of formation of the red product, from the irradiated solution, was measured at a series of temperatures in the thermostatted cell holder of the SP 1800 spectrophotometer. The results are shown in Figure 3. The kinetics of formation of the red product were investigated by plotting $\ln[a/(a - x)]$ against time t, where a = total increase in absorbance at 534 nm and x = increase in absorbance after time t (a was measured by warming an irradiated solution of tetracycline to 55 °C and maintaining it at this temperature for *ca.* 20 min, before recording the

absorbance at 534 nm). Linear plots were obtained showing that the formation of the red product obeys first order kinetics. The Table gives values of the first-order rate constant k_1 at a series of temperatures calculated from the slopes of the first-order plots. From an Arrhenius plot of the values given in the Table, the activation energy for the formation of the red product was found to be 90 kJ mol^{-1}.

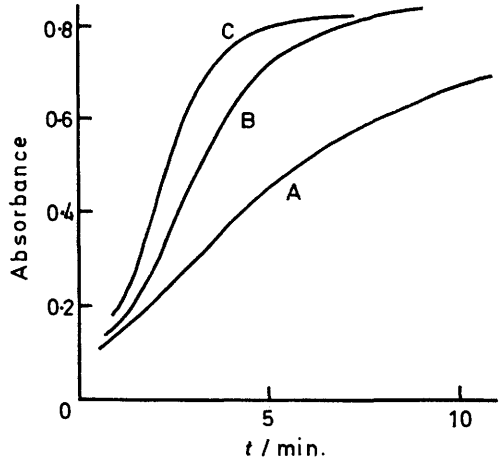


FIGURE 3 Increase in absorbance at 534 nm for the formation of the red product immediately following photolysis (through Pyrex) of tetracycline at 3 °C and pH 9.0; A, 40 °C; B, 56 °C; C, 71 °C

Using the method described in the Experimental section, the concentration of hydrogen peroxide was determined in a solution of tetracycline which had been irradiated at 3 °C and then warmed to room temperature to form the red product. From the recorder trace obtained in a typical experiment, the quantity of oxygen liberated by addition of catalase to 3 ml of the solution was 0.039 μmole. This is equivalent to 0.078 μmole H_2O_2. Since the 3 ml of solution contained 1.3 μmole of photolysed tetracycline, this corresponds to *ca.* 0.06 mole H_2O_2 formed per mole of tetracycline photolysed.

First-order rate constants for the formation of the red product, immediately following the photolysis of tetracycline in 0.1M-Na_2HPO_4 solution at 3 °C

T/K	$10^2 \times k_1$/min^{-1}
300	4.3
307	6.6
313	17.4
329	64.5
344	107.1

The effects of adding propan-2-ol and sodium azide to the solution of tetracycline prior to irradiation at *ca.* 3 °C were also investigated. Neither 20% v/v propan-2-ol nor 5 × 10^{-2}M-sodium azide had any effect on the photochemical process; on warming the solutions after photolysis, exactly the same quantity of red product was produced as was obtained in the absence of these additives.

Figure 4 shows the absorption of oxygen accompanying the formation of the red product during the irradiation, through Pyrex glass, of tetracycline (4.93 × 10^{-4}M) at pH 9.0. A total of 0.975 ml of oxygen (equivalent to 1.15 × 10^{-5} mole) was absorbed, and this corresponded to the complete photolysis of 1.0 × 10^{-5} mole of tetracycline. Thus within experimental error one mole of oxygen is absorbed per mole of tetracycline photolysed. This result, which was obtained prior to the publication of the paper by Wiebe and Moore,[2] confirms their measurements.

Properties of the Red Product.—(a) *Effect of* pH. On lowering the pH of a solution of the red product to <pH *ca.* 6, the solution became orange-yellow ($\lambda_{max.}$ 442). The red colour ($\lambda_{max.}$ 534 nm) was restored on raising the pH to *ca.* 8.5. From a plot of the absorbance at 534 nm against pH, the pK_a of the acid–base equilibrium between the orange-yellow and red forms was determined to be 7.6.

(b) *Effect of sodium dithionite.* When sodium dithionite was added to a solution of the red product the solution became pale yellow. On shaking the pale yellow solution with air, the red colour reappeared. If the minimum amount of sodium dithionite was used, the red product was recovered in *ca.* 85% yield on subsequent treatment of the solution with air. This indicates that a group in the molecule, which is responsible for the red colour, is reduced by sodium dithionite and is subsequently reoxidised by atmospheric oxygen.

(c) *Flash photolysis.* Flash photolysis of the red product in nitrogen-saturated 1 : 1 propan-2-ol–water solution at pH 9.0, in a quartz cell, gave a broad transient absorption with $\lambda_{max.}$ *ca.* 500 nm. This transient decayed with a half-life of *ca.* 2 ms. Bleaching of the solution occurred with one flash. The original colour was restored when oxygen was bubbled through the bleached solution. Flash photolysis of an oxygen-saturated solution of the red product at pH 9.0 in a quartz cell, resulted in irreversible bleaching with five photoflashes. The same solution in a Pyrex cell did not undergo photo bleaching to any marked extent.

(d) *Effect of hydrogen peroxide.* Addition of a drop of dilute hydrogen peroxide to a solution of the red product at pH 9.0 resulted in the irreversible disappearance of the red colour over a few minutes.

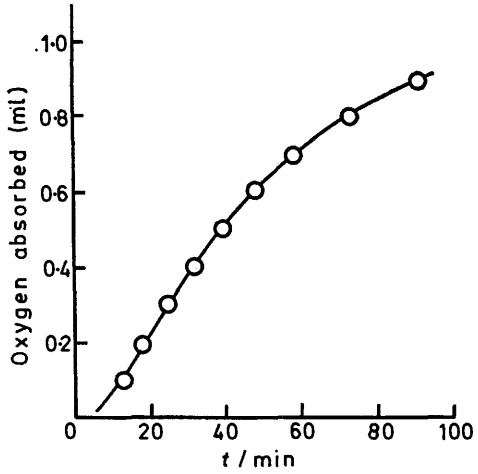
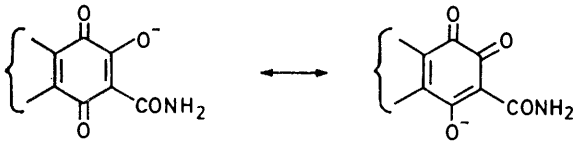


FIGURE 4 Absorption of oxygen during the photolysis (through Pyrex) of tetracycline in 0.1M-Na_2HPO_4 solution at pH 9.0

DISCUSSION

The observation of an isosbestic point in the superimposed absorption spectra of a solution of tetracycline during its photochemical alteration to give the red product suggests a simple conversion, conceivably one mole of tetracycline giving rise to one mole of red product. The fact that the red product is not produced when oxygen is absent indicates that the reaction involves

the incorporation of oxygen into the molecule. Indeed, the oxygen absorption result shows that, within experi-

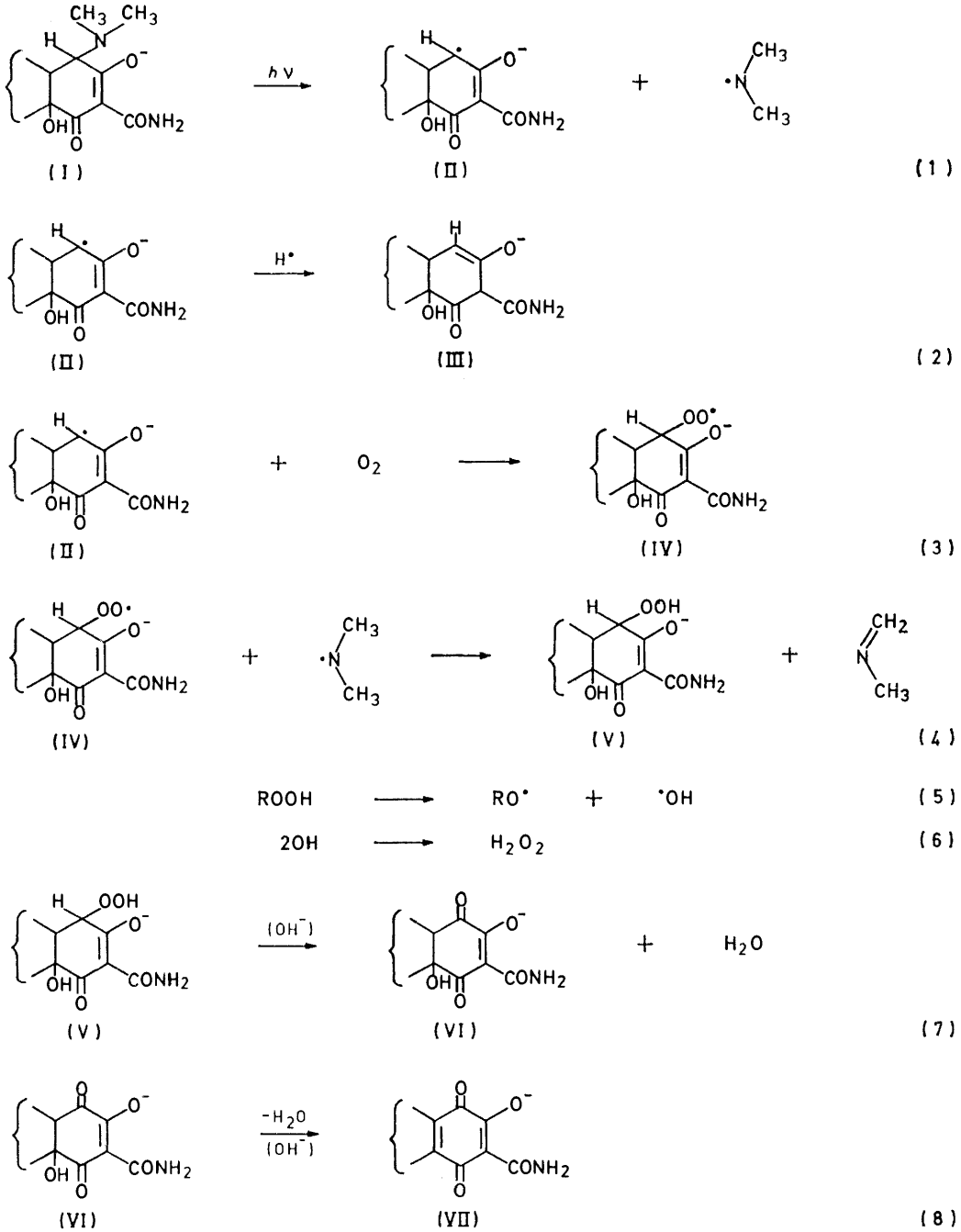


SCHEME 1

mental error, one mole of oxygen is absorbed per mole of tetracycline photochemically converted into the red product.

The low temperature irradiation experiments show that the red product is formed in two main stages, a photochemical process followed by a dark (thermal) process for which the activation energy is 90 kJ mol^{-1}. The photochemical reaction occurs more rapidly when tetracycline is irradiated through quartz, as shown by the faster fall in the absorption band at 375 nm. The red product is photolysed quite rapidly under these conditions however and does not accumulate.

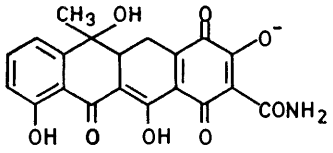
Tetracycline has three pK_a values.[2,6] pK_{a_1} (3.3) is attributed to the tricarbonyl system of ring A, pK_{a_2} (7.68) is attributed to the dimethylammonium function



of ring A, and pK_{a_3} (9.69) to the phenolic β-diketone system in the C(10)—(12) region. It follows that at pH 9 (the pH of our experiments) the state of ionisation of tetracycline will be predominantly as in Scheme 1.

Hlavka and Bitha [7] have shown that tetracycline undergoes photodeamination [equation (1)]. Under conditions favouring hydrogen atom donation to the primary radical (II), 4-dedimethylaminotetracycline (III) is formed [equation (2)]. Under our experimental conditions, in which a supply of oxygen was maintained throughout the irradiation by bubbling air or oxygen through the solution, reaction of radical (II) to form peroxyl radical (IV) is highly favoured. The high rate of reaction of radical (II) with oxygen is shown by the fact that even in the presence of 20% v/v propan-2-ol, a good hydrogen atom donor, the amount of red product formed was unchanged.

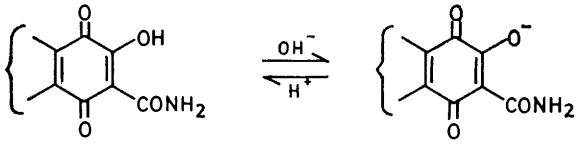
That equation (3) represents the reaction by which oxygen is added to the tetracycline molecule, rather than a process of singlet oxygen attack on tetracycline is clearly shown by the fact that azide ion, a recognised



(VII)

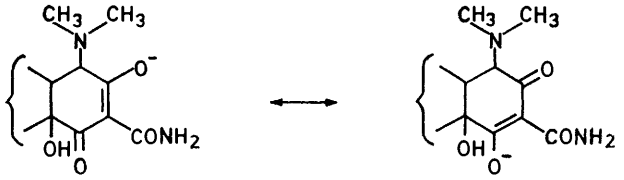
quencher of singlet oxygen,[8] does not reduce the rate of the photochemical reaction or the quantity of red product finally formed.

We believe that the formation of a hydroperoxide occurs in the next stage. One possible reaction is (4). Since hydrogen peroxide is formed only in very small amounts, decomposition of the hydroperoxide by reaction (5) followed by (6) [where ROOH represents (V)] must be a very minor process. By analogy with the base-catalysed decomposition of tetralin hydroperoxide to give α-tetralone,[9] we propose that decomposition of tetracycline hydroperoxide occurs by the base-catalysed elimination of water [equation (7)]. Since the starch iodide test, immediately following photolysis of tetracycline at 3 °C, was negative, it would appear that hydroperoxide (V) eliminates water very readily and that



orange — yellow red (9)

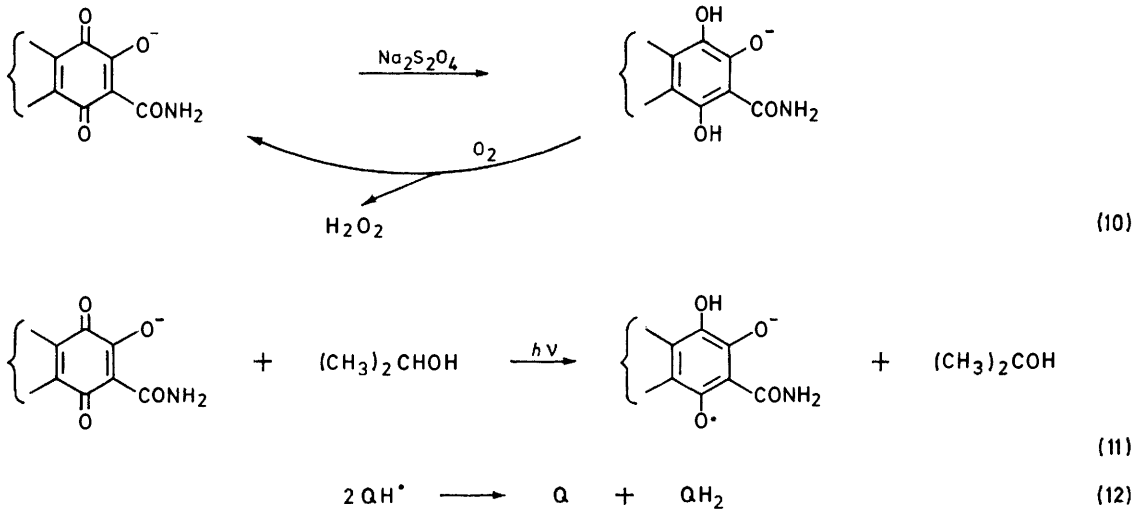
(VI) is the intermediate ($\lambda_{max.}$ 357 nm), stable for some time at 3 °C, which is the immediate precursor of the red product. This accounts for the total of *ca.* 1 mole of



SCHEME 2

oxygen consumed per mole of tetracycline photochemically changed, *i.e.* one atom of oxygen remains in the oxidised tetracycline molecule, the other is incorporated in a molecule of water.

Compound (VI) is a β-hydroxyketone which, like aldol and diacetone alcohol,[10] would be expected to undergo loss of water readily. The β-elimination of water is both acid and base catalysed.[11] We conclude that the dark reaction which leads finally to the red product is the base-catalysed elimination of water from (VI) [equation (8)]. This process is consistent with the first-order kinetics observed for the formation of the red product in the dark reaction. When the photolysis of tetracycline is carried out at room temperature or



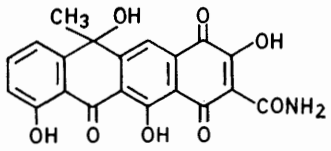
(10)

(11)

(12)

above, the dark reaction automatically follows the initial photoreaction so that the intermediate (VI) is not detected.

Thus we assign to the red product structure (VII), 4a,12a-anhydro-4-oxo-4-dedimethylaminotetracycline. This assignment is fully supported by the properties of the red compound. In the first case its reversible change to an orange-yellow form on lowering the pH



(VIII)

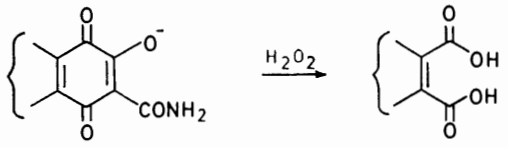
can be understood in terms of the proton equilibrium (9). In the orange-yellow form the chromophore is the *p*-benzoquinone structure, the auxochrome being the 3-OH group. Ionisation of the proton on the 3-OH group (pK_a 7.6) gives structures in Scheme 2 in which the resonance possibilities lead to delocalisation of electrons and a shift of the absorption band to longer wavelength ($\lambda_{max.}$ 534 nm) compared with the protonated form ($\lambda_{max.}$ 442 nm) which does not have corresponding canonical structures. This type of colour change with increasing pH is characteristic of hydroxy-substituted quinones.[12] Indeed, the quinone nature of the red product is further supported by the reduction with sodium dithionite. It is well known that the leuco form of a quinone, which is formed on reduction with sodium dithionite, and which generally absorbs at shorter wavelength than the parent quinone, is reoxidised by molecular oxygen to regenerate the original quinone,[13] as observed here with the red product [reaction (10)].

The quinone character of the red product is also borne out by the flash photolysis results. We assign the transient having $\lambda_{max.}$ *ca.* 500 nm to the semiquinone radical formed by hydrogen atom abstraction by the excited quinone from propan-2-ol.[14] The fact that flash photolysis of the red product in nitrogen-saturated 1 : 1 propan-2-ol–water in a quartz cell results in photobleaching, which is largely reversed by oxygen, shows that the bleaching is a photoreduction process. In this case the hydroquinone is produced by the dismutation of two semiquinone radicals [equation (12) where $QH^{\bullet}$ represents the semiquinone radical and QH_2 the hydroquinone].

Finally, our assignment of structure (VII) to the red product is substantiated by the fact that the chemical properties and spectral features of the red product are very similar to those of the closely related compound (VIII) prepared by McCormick and Jensen.[15] The irreversible disappearance of the red colour of (VII) on treatment with H_2O_2, like that of (VIII),[15] can be explained in terms of the oxidative degradation of the quinone ring [reaction (13)].

Our results fully support the conclusion of Hlavka and Bitha [7] that the deamination of tetracycline is a free radical process and not the concerted reaction (14) since (14) would exclude the possibility of attack by molecular oxygen which leads ultimately to the red product.

We have not observed the formation of corresponding



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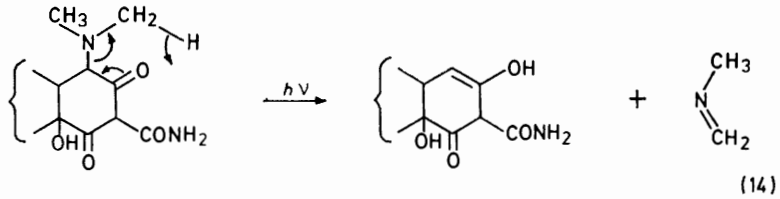

(14)

quinones with chlorotetracycline and demethylchlorotetracycline, even though the fall in the absorbance of the longest wavelength band, and the absorption of oxygen both occur on irradiating aqueous (pH 9) solutions of these derivatives. The reason for this is almost certainly the marked instability of these particular derivatives in alkaline media.[16]

We thank S.R.C. for support to A. G. R.

[8/797 *Received*, 28*th April*, 1978]

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