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Superoxidation of Retinoic Acid†

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ABSTRACT

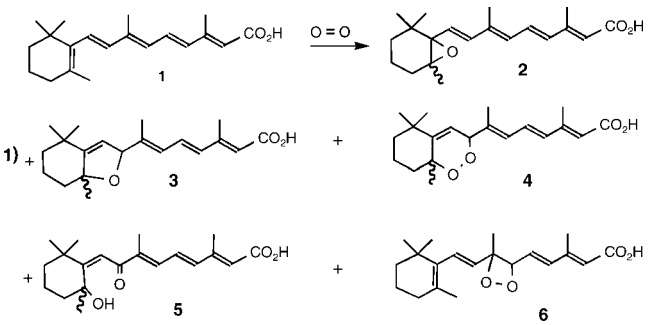
Atmospheric pressure chemical ionization mass spectroscopy (APCI-MS) was used to examine the light-induced oxidation products of retinoic acid under conditions that favor and preclude its aggregation. We observed that in conditions that favor aggregation, i.e. in aqueous solutions, retinoic acid undergoes superoxidation to yield highly oxidized species. Oxidation is limited, however, in the absence of such communication, *i.e.* when the polyene is fully solvated. From a comparison of the measured MS with that obtained from chemical oxidation of retinoic acid under conditions that promote radical oxidation and singlet oxygen–mediated oxidation, we conclude that superoxidation is mediated by reactive oxygen species other than singlet oxygen.

INTRODUCTION

Retinoic acid (**1**) and its oxidized derivatives have been found to affect fetal development, cell differentiation, metabolism and organ and cell regulation (1,2). These compounds also provide lead structures for various anticancer, anti-inflammatory and antibacterial drugs (3). In plants and food, they act as hormones, pigments, flavors, aromas and defense substances (3). Retinoic acid is also widely used as a skin treatment for acne, reverses the damage to skin by exposure to UV light and imparts elasticity to the skin, thus exhibiting anti-aging properties (4).

Under most conditions retinoic acid spontaneously reacts with oxygen (5). Its reaction with oxygen can be initiated by light, in the presence or absence of a photosensitizer, or by autoxidation and the oxidized products often show enhanced biological activity and are responsible for various aromas and tastes of food (5,6). Identification of oxygenated products is complicated by the fact that the initial species are reactive dioxetanes, peroxides, epoxides and endoperoxides, which can undergo secondary reactions and rearrangements upon chromatographic separation and identification. Furthermore, various cis/trans-, stereo- and regioisomers are often obtained.

To date, nine different products have been reported for the reaction of retinoic acid with singlet oxygen (Eq. 1) (7,8). These products include epoxide **2**, furan **3** (through rearrangement of 2), endoperoxide **4**, hydroxyketone **5**, dioxetane **6** and four degradation products (products with molecular weights lower than that of the parent retinoic acid). In each case, oxidation has been reported to cease upon addition of two oxygen atoms, the major initial products proposed to be the 5,8-endoperoxide (**4**) or the 5,6-epoxide **2** (7,8).



We recently observed that when two polyene chains are able to communicate through space they react with oxygen upon exposure to light to yield superoxidized species (9,10). Oxidation is limited, however, in the absence of such communication. We suggested that in the case of the bis-retinoids, light-initiated superoxidation is mediated by oxygen radicals or oxygen radical anions, which are formed to a greater extent. These reactive oxygen species may play a larger role in the oxidation of bis-retinoids than in the slower oxidation of specially-separated polyenes.

In aqueous solution retinoic acid has been reported to form aggregates at submicromole concentrations **7** (11–13). To date, most studies that have examined the oxidation products of retinoic acid have been performed under experimental conditions in which retinoic acid existed as monomers or tail-to-tail dimers, as shown in **8** (14,15). In order to examine the effect of aggregation on the propensity of retinoic acid to undergo light-induced oxidative damage, mass spectroscopy (MS) was used to examine irradiated solutions of retinoic acid under conditions that favor and preclude its aggregation.

MATERIALS AND METHODS

All chemicals and solvents (Aldrich, St. Louis, MO) were used as received. Absolute ethanol, dry dichloromethane (CH_2Cl_2) and deionized water were used. Irradiation was performed in borosilicate glass vials (UV cutoff of 340 nm) with λ_{max} = 350 nm light. Absorbance measurements were performed using a Jasco V-530 UV-vis spectrometer (Tokyo, Japan), MS was

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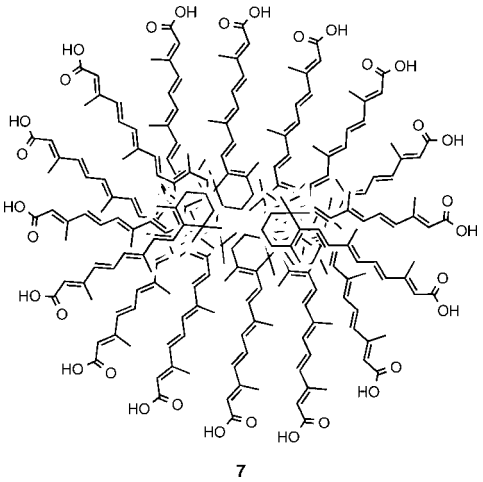
†This paper is dedicated to Professor Thomas Ebrey on the occasion of his retirement from the University of Washington.



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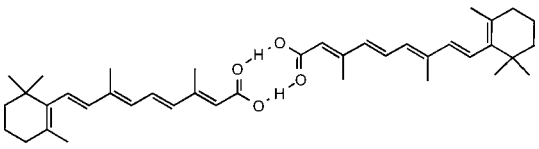

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performed with the JEOL JMS-LC mate LC/MS system (Tokyo, Japan), and the Rayonet reactor used was obtained from Rayonet Southern New England Ultraviolet Company (Branford, CT.).

RESULTS AND DISCUSSION

Figure 1 shows the UV-vis spectra of 1.58×10^{-5} *M* solutions of retinoic acid (prepared from a stock solution of ethanol by adding 20 μL of stock solution to 1 mL solvent; the concentration of retinoic acid was calculated using an extinction coefficient of 44 300 at 350 nm in ethanol [16]) in water, hexanes and ethanol. In water the measured spectrum is red-shifted by approximately 40 nm and its intensity is approximately one half that of the spectra measured in hexanes and ethanol. This red shift and decrease in extinction coefficient is indicative of retinoic acid aggregation. The retinoic acid solutions were allowed to stand under ambient white light for 4 h and then analyzed by atmospheric pressure chemical ionization (APCI)-MS for the presence of retinoic acid at m/z 301 (M+1) mass units. The hexane and ethanol solutions both were found to contain retinoic aid. On the other hand, retinoic acid was not detectable by MS in the water solution. This suggests complete decomposition of retinoic acid in the aqueous solution.

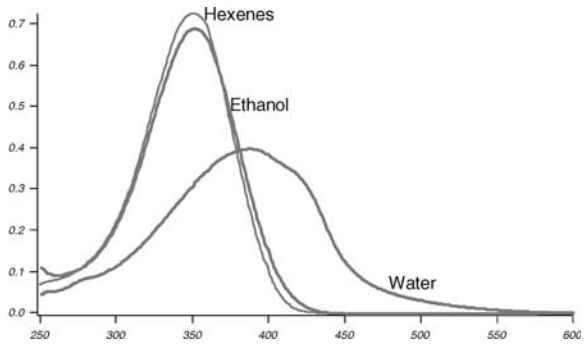


Figure 1. UV-vis spectra of 1.58×10^{-5} *M* solutions of retinoic acid in hexanes, ethanol and water, showing aggregation of retinoic acid in aqueous solutions.

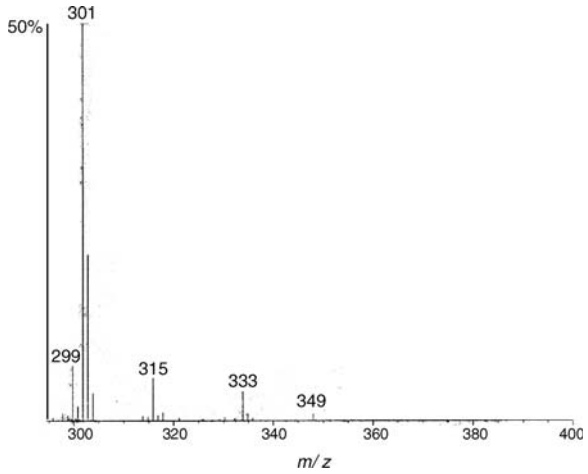
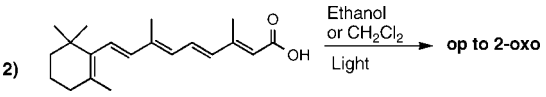


Figure 2. Atmospheric pressure chemical ionization mass spectroscopy (APCI-MS) of a solution of retinoic acid in ethanol after 24 h of irradiation with $\lambda_{max} = 400$ nm light, showing the maximum addition of up to two oxygens.

For further MS analysis, more concentrated solutions of retinoic acid where prepared in ethanol and CH_2Cl_2 (7.8 mM) and in a mixture of water and ethanol (60% water, 40 % ethanol; 0.007 g in 5–7 mL). The solutions were irradiated with $\lambda_{max} = 350$ nm light in borosilicate glass using a Rayonet reactor for 24 h. Figure 2 shows the APCI-MS of the ethanol solution after irradiation (Eq. 2). The peak at m/z 301 corresponds to retinoic acid (M+1). The spectrum has a second signal at m/z 315, which corresponds to the mass of retinoic acid (**1**) plus one oxygen atom (M–1). The third peak at m/z 333 represents the bis-oxygen adduct of **1** (M+1). In some runs a small peak was found at m/z 347, which corresponds to the addition of three oxygen atoms. However, for the majority of runs no peaks were found above m/z 333. This suggests that in most cases oxidation of **1** stops after the addition of two oxygen atoms. Finally,



an additional peak was found at m/z 299, which is presumably the result of fragmentation of a higher mass species upon ionization.

The measured MS spectra of the CH_2Cl_2 solution after irradiation was similar to that shown in Fig. 2, with a maximum peak at +32 Da, which corresponds to the parent ion plus two oxygen atoms, and a smaller peak at +16 Da, which corresponds to the addition of one oxygen atom to the parent ion. The peaks located at 16 Da and 32 Da are consistent with reports of formation of the 5,6-epoxide and the 5,8-endoperoxide, respectively (7,8) . In both the CH_2Cl_2 and ethanol solutions there is little communication between neighboring polyene systems (13,14).

Figure 3 shows the APCI-MS of the aqueous ethanolic solution of retinoic acid after irradiation (Eq. 3). The peak at m/z 301 corresponds to the parent mass of retinoic acid. Subsequent peaks were found at increments of 16 Da, which corresponds to the addition of one oxygen atom. The spectrum shows the maximum m/z at 379 (M–1), which corresponds to the addition of five oxygen atoms. Retinoic acid exists mainly as aggregates under

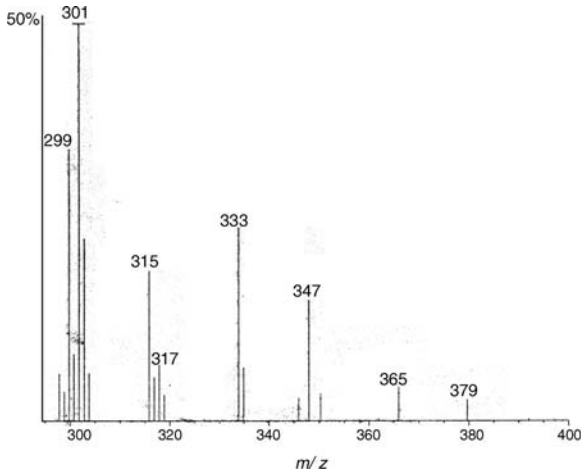
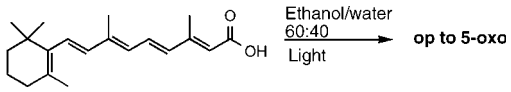


Figure 3. Atmospheric pressure chemical ionization mass spectroscopy (APCI-MS) of a solution of retinoic acid in 60:40 ethanol:water after 24 h of irradiation with $\lambda_{max} = 400$ nm light, showing the maximum addition of up to five oxygens.

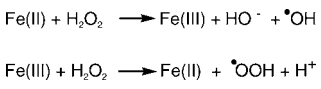
these reaction conditions. Closer inspection of the MS data reveals peaks at m/z 315 and 317, which correspond to two mono-oxygen adducts, M–1 and M+1, respectively. The two different peaks could have arose from fragmentation of a larger molecular weight ion or could represent two different mono-oxygen adducts. The ratio of peaks in the aqueous oxidation also differs from that of nonaqueous oxidations. Peaks corresponding to the oxygen adducts (315, 333, 347, *etc*.) are more intense relative to the parent ion. The peak at m/z 299 is more intense and there are

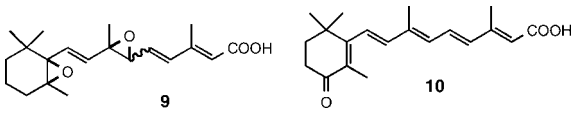


several peaks at <m/z 301, which correspond to several retinoic acid degradation products (data not shown).

The lifetime of singlet oxygen in CH_2Cl_2, ethanol and water are 59 μs (17), 12 μs (17) and 3.5 μs (18), respectively. Thus, if singlet oxygen were the sole oxidizing species involved, one would expect an approximately 30-fold increase in the rate of oxidation of retinoic acid in CH_2Cl_2 compared with water and a six-fold increase in the rate of oxidation of retinoic acid in ethanol compared with water. Conversely, we observe the fastest, and more exhaustive, oxidation in water under our experimental conditions.

In the presence of an iron catalyst, hydrogen peroxide forms hydroxyl radicals (•OH) and other relative oxygen species according to (Eq. 4). Thus, a crystal of iron(II) sulfate was added to a solution of retinoic acid (0.007 g) in 3 mL of a 90:10 solution of ethanol:hydrogen peroxide (30% hydrogen peroxide solution). MS analysis of the reactant mixture after 2 h produced a spectrum that revealed the addition of up to five oxygens, which was nearly identical to that obtained from direct irradiation of retinoic acid aqueous solutions (Fig. 3). The 5,6-epoxide **2**, and bis-epoxide **9** have been identified as the major products of radical oxidation of retinoic acid, along with minor amounts of the 4-oxo-retinoic acid,





10 (19,20). MS analysis enabled the detection of additional, presumably reactive, poly-oxidized species of retinoic acid.

In the presence of molybdinate, hydrogen peroxide disproportionates to form singlet oxygen. Thus, sodium molybdinate was added to a solution of retinoic acid (0.007 g) in 3 mL of a 90:10 solution of ethanol:hydrogen peroxide (30% hydrogen peroxide solution). MS analysis of the reactant mixture after 2 h produced a spectrum that revealed the addition of up to two oxygens, similar to that obtained from direct irradiation of retinoic acid in ethanol or CH_2Cl_2 (Fig. 2).

CONCLUSION

We have shown that aqueous solutions of retinoic acid, in which the acid aggregates, undergo light-initiated superoxidation to yield highly oxidized species. Oxidation is limited, however, in the absence of aggregation, when the polyene is fully solvated. We provide evidence that superoxidation may be a result of the increased propensity of these aggregates to form reactive oxygen species such as hydroxyl radicals upon exposure to light. These conclusions have relevance to the mechanism of the biological activity of topical retinoic acid. Furthermore, these observations may explain why retinoic acid–binding proteins are present in concentrations that exceed retinoic acid (21), and why upon exposure to UV light the concentration of retinoic acid–binding proteins decreases as much as 50%–90% (22), while the concentration of retinoic acid increases in the exposed areas (23). Rapid, high-affinity binding of retinoic acid at the skin surface may be necessary to prevent it from aggregating and undergoing superoxidation. Programmed release of retinoic acid and its subsequent aggregation may allow it to undergo light-initiated superoxidation to form its biologically active oxidized analogues as needed.

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