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Structure-Activity Relationships for the Formation of Secondary Radicals and Inhibition of Keratinocyte Proliferation by 9-Anthrones

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SUMMARY

The biological properties of tumor-promoting and antipsoriatic 9-anthrones have been hypothesized to be mediated by free radical products such as the corresponding 9-anthron-10-yl radicals or by $O_2^{\cdot}$, $^{\cdot}OH$, and other persistent secondary radicals that are formed in the skin after topical treatment with 9-anthrones. To gain additional insights into the possible role of reactive oxygen or secondary radicals in mediating the biological effects of 9-anthrones, we have used EPR spectroscopy to investigate the formation of these species by a series of 9-anthrones or 9-anthrone dimers with known tumor-promoting and antipsoriatic activities. The effect of the 9-anthrones on keratinocyte proliferation *in vitro* was also investigated. 5,5-Dimethyl-1-pyrroline *N*-oxide was used as a spin trap to detect reactive oxygen-centered radicals in aqueous buffer/dimethylsulfoxide solutions. Superoxide was trapped during the autoxidation of most of the 9-anthrones. For 9-anthrones that generated no detectable superoxide, evidence of anthronyl-peroxyl radical formation was found instead. In the presence of Fe^{3+} complexed to EDTA, but not diethylenetriaminepentaacetic acid, the hydroxyl radical was produced by all of the 9-anthrones. 9-Anthrone dimers produced oxygen-centered radicals only weakly or not at all. Direct EPR was used to detect 9-anthrone-derived secondary radicals in keratinocyte suspensions or in dimethylsulfoxide solutions. These radicals were similar to those previously reported to occur in skin after topical treatment with the antipsoriatic drug anthralin (1,8-dihydroxy-9-anthrone). In contrast to the ubiquitous ability of the 9-anthrones to generate reactive oxygen radicals, only the hydroxy-substituted 9-anthrones or their dimers possessed significant secondary radical-forming ability. The ability of the 9-anthrones or dimers to form secondary radicals in keratinocytes was found to correlate with their *in vitro* inhibition of keratinocyte proliferation. The data suggest the possible importance of reactive dimeric intermediates in mediating the biological effects of the 9-anthrones.

Certain hydroxy-substituted 9-anthrones such as anthralin (1,8-dihydroxy-9-anthrone, dithranol, or cignolin) (Fig. 1, **1**) are effective in the topical treatment of psoriasis (1). Significant side effects of 9-anthrone treatment include severe skin irritation and inflammation (2–8) and staining of the skin (2, 4). Antipsoriatic 9-anthrones have also been shown to be tumor promoters in mouse skin (9–18). Although they have been in clinical use for >100 years, the chemical mechanisms underlying both the therapeutic and adverse effects of the 9-anthrones are presently uncertain.

Under physiological conditions, 9-anthrones are susceptible to rapid photo- or autoxidation at the 10-position to form the corresponding anthraquinones, dimers (Fig. 1, **4** and **5**), and other persistent free radical products (19, 20). Molecular oxygen is reduced to superoxide anion radical during this oxidation process (Fig. 1) (21–24). Because the oxidation products (Fig. 1, **4** and **5**) are not effective in the treatment of psoriasis (25) and are not tumor promoters (10, 26), it has been suggested that the 9-anthron-10-yl radical (Fig. 1, **3**), or the reactive oxygen species produced from the oxidation process, is the mediator of these biological responses (4, 18, 21, 27–31). Recently, however, we have used the EPR spin-trapping technique (32) and direct EPR[1] to detect and study the 9-anthron-10-yl radical formed by a variety of 9-anthrones, and we have found that the ability to generate this radical does not correlate with the known clinical activities of the 9-anthrones.

In the present report we have extended our previous studies to address the possible role that oxygen-centered radicals or "anthralin brown" secondary radicals may play in mediating the biological effects of this class of compounds. DMPO was used as a spin trap to detect reactive oxygen-centered radicals generated in aqueous/Me_2SO solutions by a variety of 9-an-

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[1] Motten, A.G., R.H. Sik, C.F. Chignell, and J.P. Hayden. An EPR study of free radicals formed by antipsoriatic and tumor-promoting 9-anthrones in non-polar solvents. Manuscript in preparation.

ABBREVIATIONS: DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; Me_2SO, dimethylsulfoxide; DTPA, diethylenetriaminepentaacetic acid; SOD, superoxide dismutase.

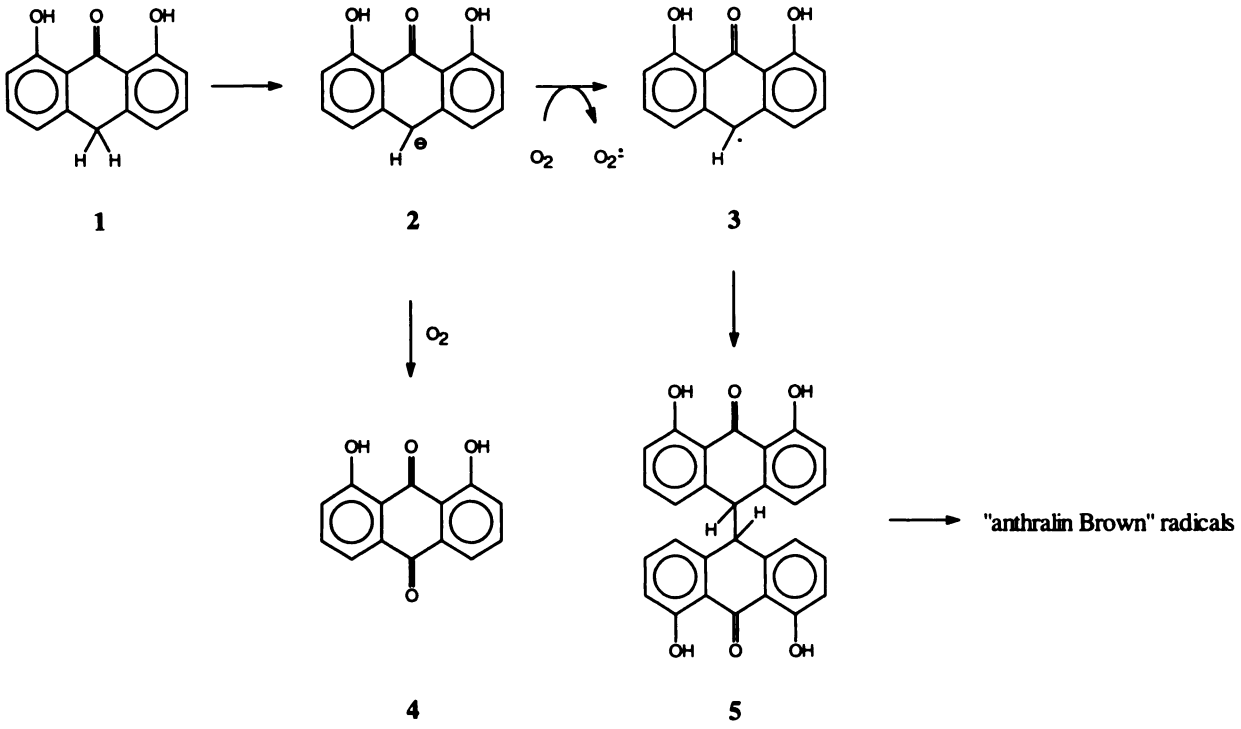
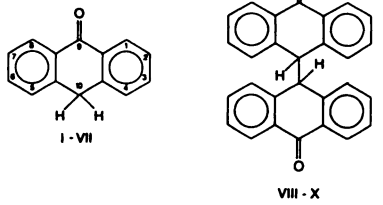


Fig. 1. Proposed mechanisms for oxidative degradation of anthralin.

TABLE 1
Structure and biological activity of 9-anthrone derivatives



Compound	Tumor promoter	Antipsoriatic	Ref.
I. 1,8-Dihydroxy-9-anthrone (anthralin)	Yes	Yes	9, 25
II. 1-Hydroxy-9-anthrone	Yes	Yes	11, 25
III. 1,8-Dihydroxy-3-methyl-9-anthrone (chrysarobin)	Yes	Yes	14, 25
IV. 3-Methyl-1,6,8-trihydroxy-9-anthrone (emodin anthrone)	No	ND[a]	17
V. 9-Anthrone	No	No	10, 25
VI. 1-Amino-9-anthrone	ND	No	25
VII. 1,8-Dichloro-9-anthrone	ND	No	25
VIII. Anthrone dimer	ND	ND	
IX. Anthralin dimer	No	No[b]	10, 25
X. Chrysarobin dimer	ND	ND	

[a] ND, not determined.
[b] Inhibits proliferation of human keratinocytes *in vitro* (19, 52).

thrones or 9-anthrone dimers. Superoxide was trapped during the autoxidation of most of the 9-anthrones. For 9-anthrones that generated no detectable superoxide, evidence of anthronyl-peroxyl radical formation was found instead. In the presence of Fe^{3+} complexed to EDTA, but not DTPA, the hydroxyl radical was produced by all of the 9-anthrones, indicating the formation of superoxide and hydrogen peroxide. 9-Anthrone dimers produced oxygen-centered radicals only weakly or not at all. Direct EPR was used to detect 9-anthrone-derived secondary radicals in keratinocyte suspensions or in Me_2SO solutions. These radicals were similar to those previously reported to occur in skin after topical treatment with anthralin (1,8-dihydroxy-9-anthrone) (33, 34). In contrast to the ubiquitous ability of the 9-anthrones to generate reactive oxygen radicals, only the hydroxy-substituted 9-anthrones or their dimers possessed significant secondary radical-forming ability. The ability of the 9-anthrones or dimers to form secondary radicals in keratinocytes was found to correlate with their *in vitro* inhibition of keratinocyte proliferation. The data suggest the possible importance of reactive dimeric intermediates in mediating the biological effects of the 9-anthrones.

Experimental Procedures

Materials. Anthrones II-IV and VI-VII (Table 1) were prepared by reduction of the corresponding anthraquinones with stannous chloride and HCl in glacial acetic acid, according to the method of Auterhoff and Scherff (35). Anthrone dimer (Table 1, compound VIII) was prepared by oxidation of anthrone with pyridine *N*-oxide and ferrous sulfate in glacial acetic acid, according to the method of Haginiwa *et al.* (36). Anthralin dimer (Table 1, compound IX) and chrysarobin dimer (Table 1, compound X) were prepared by oxidation of the anthrone with ferric chloride in either glacial acetic acid (35) or ethanol (37), respectively. Products were purified by recrystallization or column chromatography when necessary. Identity and purity (>98%) of synthetic compounds were confirmed by NMR and/or by mass spectrometry and high pressure liquid chromatography. DMPO was purchased from Sigma Chemical Co. (St. Louis, MO) and was purified by vacuum distillation before use. Catalase was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Buffers were treated with Chelex 100 resin to remove metal impurities (Bio-Rad Laboratories, Hercules, CA). All other chemicals and reagents were purchased from commercial sources and used as received. Stock solutions (2.0 mM) of anthrones were prepared in Me_2SO and used within 15 min.

EPR experiments. EPR spectra were acquired with a Bruker ESP300, IBM ER 200D-SRC, or Varian E109 spectrometer operating in the X-band frequency range. All experiments were conducted at ambient room temperature (~23°) with air-saturated buffers, 20-mW

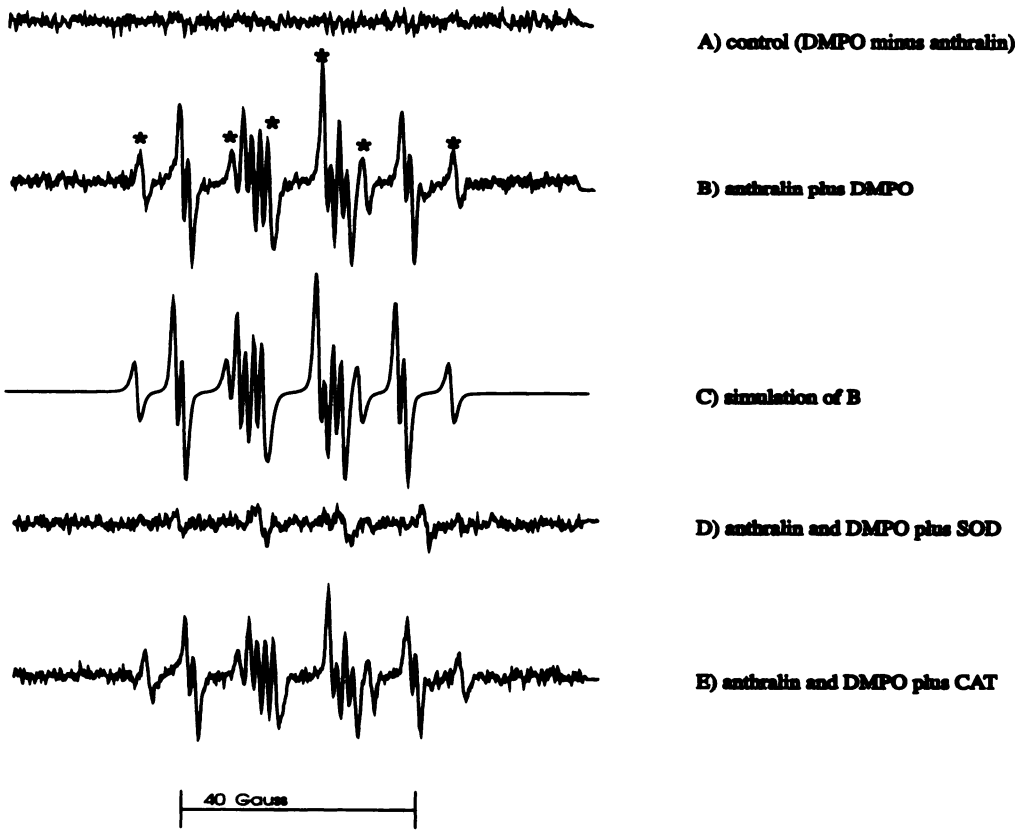


Fig. 2. EPR spectra of DMPO spin adducts generated by autoxidation of anthralin in air-saturated phosphate buffer, pH 7.5, containing 50% Me_2SO. Samples contained 500 μl of 50 mM sodium phosphate buffer, pH 7.5, 20 μl of DMPO, and 500 μl of 0.8 mM anthralin in Me_2SO. Scans were initiated immediately after mixing of the sample components. Spectra were acquired at 9.8 GHz with 20-mW microwave power, 100-kHz modulation frequency, 0.33-G modulation amplitude, 250-msec time constant, 1.6×10^4 gain, and 4.0-min scan time. Where indicated, the SOD DMPO/$^{\cdot}$R concentration was 450 units/ml and the catalase (*CAT*) concentration was 650 units/ml. DMPO/$^{\cdot}$OOH, a_N = 13.58 G, a_β^H = 10.79 G, a_γ^H = 1.33 G. a_N = 15.71 G, a_β^H = 22.32 G. *, Signals produced by DMPO/$^{\cdot}$R.

microwave power, and 100-kHz magnetic field modulation frequency. Other instrument parameters and experimental conditions are indicated in the figure legends for specific experiments. Spectral simulations were performed on a personal computer utilizing a program written by David R. Duling (Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, Research Triangle Park, NC). The hyperfine splitting constants quoted in the text and figure legends were obtained from simulations of the experimental data. The standard deviation of the hyperfine splitting values obtained from three or more individual experiments was typically <0.1 G.

Cell culture and proliferation experiments. BALB MK keratinoctyes were provided by Dr. Thomas E. Eling (National Institute of Environmental Health Sciences, Research Triangle Park, NC) and were cultured at 37° with 5% CO_2, in modified minimal essential medium containing L-glutamine and 50 μM $CaCl_2$ (JRH Biosciences, Lenexa, KS) and supplemented with 3.0% dialyzed fetal bovine serum (GIBCO Laboratories, Grand Island, NY), 10 ng/ml epidermal growth factor (Biomedical Technologies Inc., Stoughton, MA), and 44 μg/ml ampicillin (Sigma) (complete medium). For EPR experiments confluent cultures were harvested by treatment with 0.1% trypsin (Sigma) in phosphate-buffered saline. Cells were then washed and suspended in 50 mM sodium phosphate, pH 7.5, containing 150 mM NaCl, 5.0 mM glucose, and 1.0 mM DTPA.

Cell proliferation was measured by incorporation of [^{3}H]thymidine into DNA. Cells were seeded into 24-well dishes at a density of 17,500 cells/well and were grown until they reached approximately 50% confluence (typically 2 days). The complete medium was then replaced with 1 ml of serum-free medium and the cells were treated with various doses of the indicated 9-anthrone. Stock solutions of 9-anthrones were dissolved in Me_2SO (10 mM) immediately before use. The final concentration of Me_2SO was 0.1% for all treatments. After 1.5 hr the serum-free medium containing the 9-anthrones was replaced by 1 ml of complete medium containing 3 μCi of [^{3}H]thymidine (65 Ci/mmol), and the cells were incubated for 20 hr. The medium containing [^{3}H]thymidine was then removed, and the cells were rinsed with 1 ml of phosphate-buffered saline and then extracted with 1 ml of cold 5% trichloroacetic acid for 15 min at 4°. After the trichloroacetic acid solution was removed and the cells were rinsed twice with cold 95% ethanol, the cells were dissolved by addition of 500 μl of 200 mM NaOH, containing 0.1% sodium dodecyl sulfate, and gentle agitation for 15 min at room temperature. Finally, the solutions were neutralized by addition of 100 μl of 1.0 N HCl, and the amount of ^{3}H in an 120-μl aliquot was determined by scintillation counting.

Results

When anthralin was allowed to autoxidize in air-saturated solutions (50 mM sodium phosphate, pH 7.5, 1.0 mM DTPA, 50% Me_2SO) in the presence of DMPO, the EPR spectrum shown in Fig. 2B was obtained. Analysis of the hyperfine coupling constants [by comparison with parameters of previously reported spin adducts (38) and computer simulation (Fig. 2C)] indicated the presence of two nitroxide radical species, i.e., the DMPO adduct of superoxide anion radical and the DMPO adduct of a carbon-centered radical. In the presence of SOD, no spin adducts were detected (Fig. 2D). In contrast, catalase had no effect on the production of either radical species (Fig. 2E).

Whereas the appearance of the DMPO adduct of superoxide is expected (see Fig. 1), the origin of the DMPO/$^{\cdot}$R (carbon-centered radical adduct) signal was unclear. Most carbon-centered radical adducts of DMPO show very similar hyperfine

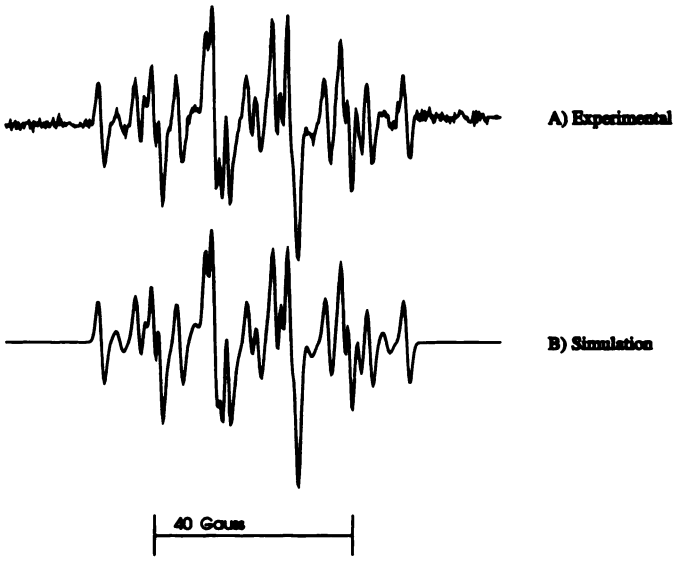


Fig. 3. EPR spectra of DMPO spin adducts generated by autoxidation of anthralin in air-saturated sodium phosphate buffer, pH 7.5, containing 50% [^{13}C]Me_2SO. Experimental conditions were identical to those described for Fig. 2 except as follows: modulation amplitude was 0.66 G, time constant was 500 msec, and scan time was 8 min. The scan presented was obtained ~10 min after mixing of the reaction components and shows contributions from small amounts of DMPO/$^{\cdot}$[^{12}C]CH_3, from [^{12}C]Me_2SO used to prepare the stock solution of anthralin, as well as small amounts of DMPO/$^{\cdot}$OH. DMPO/$^{\cdot}$[^{13}C]CH_3, a_N = 15.80 G, a_β^H = 22.50 G, a_β^{13C} = 7.50 G, relative area = 60; DMPO/$^{\cdot}$[^{12}C]CH_3, a_N = 15.70 G, a_β^H = 22.30 G, relative area = 10; DMPO/$^{\cdot}$OOH, a_N = 13.68 G, a_β^H = 10.89 G, a_γ^H = 1.33 G, relative area = 177; DMPO/$^{\cdot}$OH, a_N = 14.60 G, a_β^H = 13.70 G, a_γ^H = 0.31 G, relative area = 25.

coupling constants. Therefore, it was not possible to unequivocally identify the carbon-centered radical species shown in Fig. 2B on the basis of its EPR parameters alone. It seemed possible that the adduct could be due to trapping of the primary anthralin radical (Fig. 1, 3), but the formation of this radical would not be expected to be affected by SOD, as shown in Fig. 2D. The Me_2SO in the buffer was an additional possible source of carbon-centered radicals. Therefore, to determine the source of the carbon-centered radical, experiments were repeated using [^{13}C]Me_2SO. The EPR spectrum obtained displayed an additional doublet splitting of the carbon-centered signal of 7.50 G due to the 0.5 spin possessed by the ^{13}C nucleus (Fig. 3). This result clearly shows that Me_2SO is the source of the carbon-centered adduct.

Production of the methyl radical by reaction of hydroxyl radical with Me_2SO is a well known phenomenon (39). Evidence of anthralin-dependent hydroxyl radical formation in phosphate buffer has been reported previously (31, 40). The mechanism of its formation was proposed to be via the metal-catalyzed Haber-Weiss reaction. However, the experiments presented in Fig. 2 were performed in the absence of redox-active metals. Anthralin is also a strong reductant. Therefore, the possibility that it may be capable of directly reducing the H_2O_2 formed by dismutation of superoxide to produce hydroxyl radical must be considered. However, the results of experiments conducted in chelexed buffer and in the presence of DTPA and SOD, as shown in Fig. 2D, indicate that anthralin cannot directly reduce hydrogen peroxide to produce hydroxyl radical. Thus, an alternate explanation for the formation of the methyl radical adduct shown in Fig. 2 must be sought.

The DMPO/$^{\cdot}$OOH adduct decays rapidly, with a half-life on the order of 50 sec (41). One of the decomposition products of DMPO/$^{\cdot}$OOH is the hydroxyl radical (42). Therefore, the most likely source of the DMPO/$^{\cdot}CH_3$ adduct signal observed in Fig. 2 is via the decomposition of DMPO/$^{\cdot}$OOH to form hydroxyl radical, which in turn reacts with Me_2SO to form methyl radical. Thus, the hydroxyl radical formed in the experiments described above is an artifact of the spin-trapping system containing DMPO. However, as mentioned above, Müller and co-workers (31, 40) have reported anthralin-dependent generation of hydroxyl radical in phosphate buffer.

To explore possible mechanisms of anthralin-dependent hydroxyl radical formation that may be relevant in biological systems and to investigate the role of metals in these mechanisms, additional experiments were conducted. When the experiments in Fig. 2 were repeated in the presence of 10 μM Fe^{3+}/EDTA, an intense DMPO/$\cdot CH_3$ EPR signal, indicating hydroxyl radical formation, was observed, in confirmation of the previous proposal of other workers (31, 40) (Fig. 4A). Addition of SOD to the incubation mixture increased the intensity of the hydroxyl radical adduct signal (Fig. 4B), indicating that anthralin can directly reduce Fe^{3+}/EDTA. However, no EPR signals were seen when the Fe^{3+} was complexed with DTPA instead of EDTA (Fig. 4C), demonstrating that anthralin is not a powerful enough reductant to reduce either H_2O_2 or the Fe^{3+}/DTPA complex. Catalase completely prevented the formation of the DMPO/$^{\cdot}CH_3$ signal in the presence of Fe^{3+}/EDTA, confirming the involvement of hydrogen peroxide in hydroxyl radical formation (Fig. 4D).

To determine the structural requirements for oxygen radical generation by 9-anthrones and to assess the importance of these radicals in mediating the biological effects of 9-anthrones, a series of 9-anthrones, as well as several 9-anthrone dimers, were examined (Table 1). When these compounds were tested for their abilities to produce oxygen-centered radicals in the absence of redox-active metals, it was found that several of them [1-hydroxy-9-anthrone, 1,8-dihydroxy-3-methyl-9-anthrone (chrysarobin), and anthralin dimer] produced the same DMPO spin adducts as found with anthralin (Fig. 5). The unsubstituted 9-anthrone dimer also produced a weak spectrum, but no signals were observed with the dimer of 1,8-dihydroxy-3-methyl-9-anthrone (chrysarobin dimer) (Fig. 5).[2] Surprisingly, two of the compounds [the unsubstituted 9-anthrone and 3-methyl-1,6,8-trihydroxy-9-anthrone (emodin anthrone)] produced spectra characteristic of the DMPO spin adduct of the hydroxyl radical (DMPO/$^{\cdot}$OH, a_N = 14.40 G, a_β^H = 13.50 G, and a_γ^H = 0.70 G), whereas two of the compounds (1-amino-9-anthrone and 1,8-dichloro-9-anthrone) showed significant amounts of superoxide, methyl, and hydroxyl spin adducts (Fig. 5).

It seemed highly unlikely that the DMPO/$^{\cdot}$OH adduct could be due to trapping of the hydroxyl radical, because the reaction mixture contained 50% Me_2SO, which would scavenge the vast majority of any hydroxyl radicals formed. Consistent with this notion, neither SOD (450 units/ml) nor catalase (650 units/ml) had any effect upon the formation of the EPR signal produced by the unsubstituted 9-anthrone or emodin anthrone (data not shown). Analysis of the structural similarities between the compounds that produce these DMPO/$^{\cdot}$OH signals suggests that these adducts are produced by those compounds that cannot stabilize the primary 9-anthron-10-yl radical well

[2] In the case of the unsubstituted 9-anthrone and its dimer, an extremely intense DMPO/$^{\cdot}$OH signal was also observed when incubations were conducted in the presence of bright sunlight (i.e., sunlight filtered through window glass, with λ of >300 nm). All of the 9-anthrones, their corresponding anthraquinones, and dimers produced this signal upon higher intensity irradiation with a xenon lamp. Evidence obtained with [^{17}O]H_2O and $^{17}O_2$ indicates that the signal is due to addition of H_2O to DMPO, followed by O_2-dependent oxidation. These data will be presented in a separate report. To avoid this effect, experiments using these compounds were conducted with the window shades drawn.

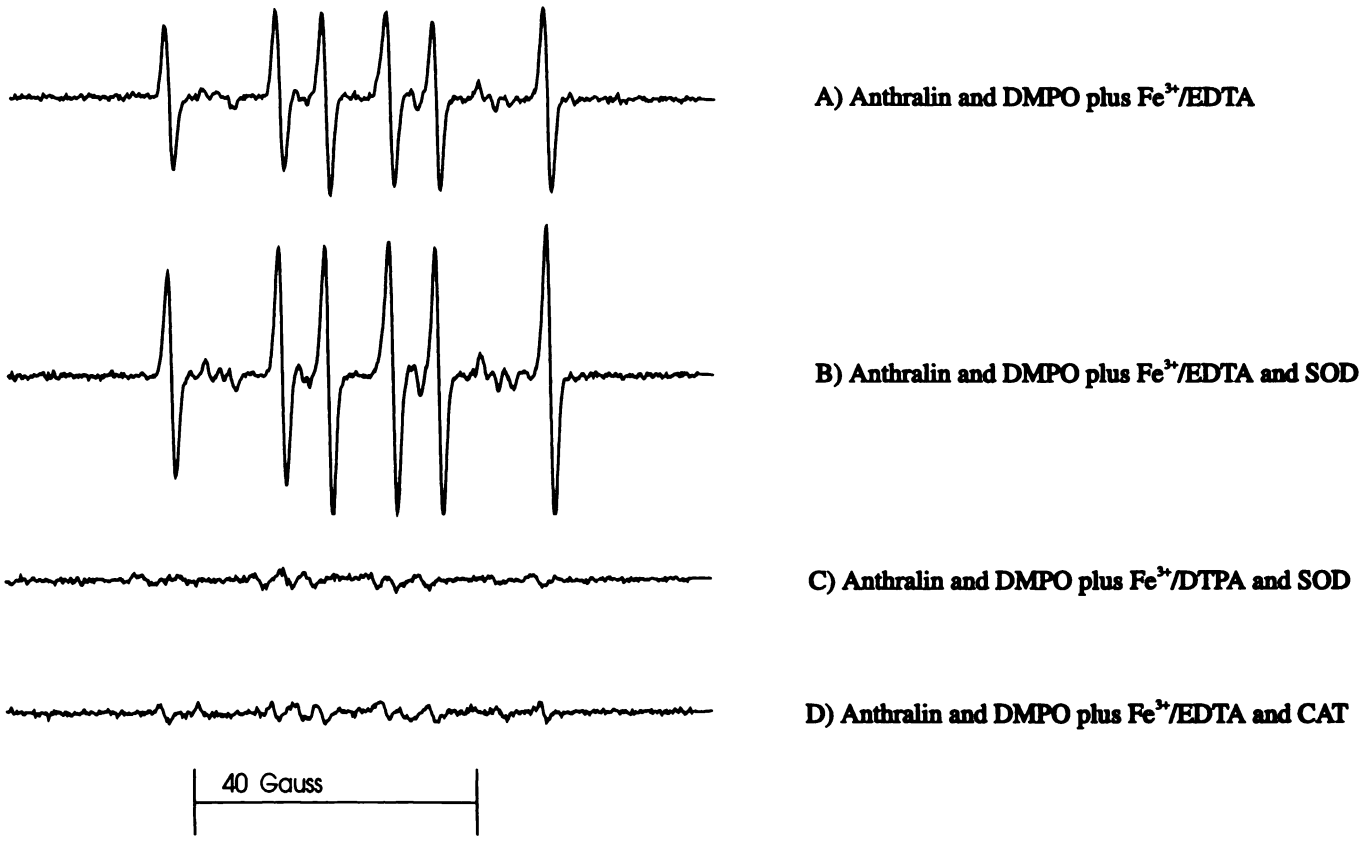


Fig. 4. EPR spectra of DMPO spin adducts produced by autoxidation of anthralin in air-saturated sodium phosphate buffer, pH 7.5, containing 50% Me_2SO and Fe^{3+} complexes. Experimental conditions were identical to those described for Fig. 2. DMPO/$^{\cdot}CH_3$, a_N = 15.71 G, a_β^H = 22.22 G. Chelator concentration was 1.0 mM and total Fe^{3+} concentration was 10 μM. *CAT*, catalase.

(Fig. 6). We propose that these more reactive 9-anthron-10-yl radicals can react with molecular oxygen at a significant rate to form the corresponding 9-anthron-10-peroxyl radical. This radical is trapped by DMPO, and the resulting spin adduct in turn decomposes very rapidly to produce DMPO/$^{\cdot}$OH and the corresponding anthraquinone (Fig. 7). Evidence to support this reaction mechanism as well as other mechanisms of artifactual nitroxide radical production from DMPO in the presence of anthrones and anthraquinones will be presented in a separate report.[3] The rapid reaction of superoxide with the peroxyl radical (to form the hydroperoxide and molecular oxygen), compared with the reaction of superoxide with DMPO, may also explain the lack of observation of DMPO/$^{\cdot}$OOH in experiments with the unsubstituted 9-anthrone and emodin anthrone.

Structure-activity experiments with the 9-anthrones and dimers were also conducted in the presence of Fe^{3+}/EDTA. The results of these studies showed that all of the 9-anthrones tested were capable of generating the methyl radical (indicating hydroxyl radical formation) by a catalase-inhibitable mechanism (Fig. 8; results with 650 units/ml catalase not shown). The presence of Fe^{3+}/EDTA allowed the detection of superoxide production from all of the 9-anthrones except the 1,8-dichloro compound. Consistent with results presented in Fig. 5, only weak signals or no signals were observed in experiments with the 9-anthrone dimers.

The results presented in Fig. 8 demonstrate that, although the production of superoxide by the unsubstituted 9-anthrone or 3-methyl-1,6,8-trihydroxy-9-anthrone was not detected in Fig. 5, this species, as well as hydrogen peroxide, is produced in the presence of these compounds. An alternate, but perhaps less likely, mechanism for the production of hydroxyl radical is via reduction of the subsequently formed 9-anthron-10-hydroperoxide by Fe^{2+}. Because catalase may also (albeit more slowly) decompose organic hydroperoxides, this reaction might also be expected to be catalase sensitive. Regardless of the mechanism, these results demonstrate that the hydroxyl radical can be formed by all of the 9-anthrones tested in the presence of redox-active iron and molecular oxygen.

Although the experiments described above demonstrate that all of the 9-anthrones autoxidize to produce oxygen-centered radicals without a requirement for metabolism, we wished to confirm that these radicals were also formed in skin cells, a probable target of 9-anthrone effects. In addition, we wished to see whether other secondary radicals, such as anthralin brown (Fig. 1) or related radicals, which might require metabolism of 9-anthrones or their autoxidation products, could be detected in the presence of cells. Therefore, EPR spin-trapping experiments in keratinocyte suspensions were also conducted.

When anthralin and DMPO were incubated in the presence of keratinocytes, a four-line signal corresponding to the DMPO/$^{\cdot}$OH adduct and a broad singlet similar to that which has been attributed to anthralin brown were observed by EPR (Fig. 9A). In the absence of DMPO, only the broad singlet signal was observed (data not shown). As in the previous experiments, it seemed unlikely that the DMPO/$^{\cdot}$OH signal could be due to trapping of the hydroxyl radical, because the hydroxyl radical would be expected to react preferentially with Me_2SO (41, 42). However, the superoxide anion radical adduct

[3] Hayden, P. J. and C. F. Chignell. DMPO/$^{\cdot}$OH artifacts formed during spin trapping experiments with 9-anthrones and 9,10-anthraquinones. Manuscript in preparation.

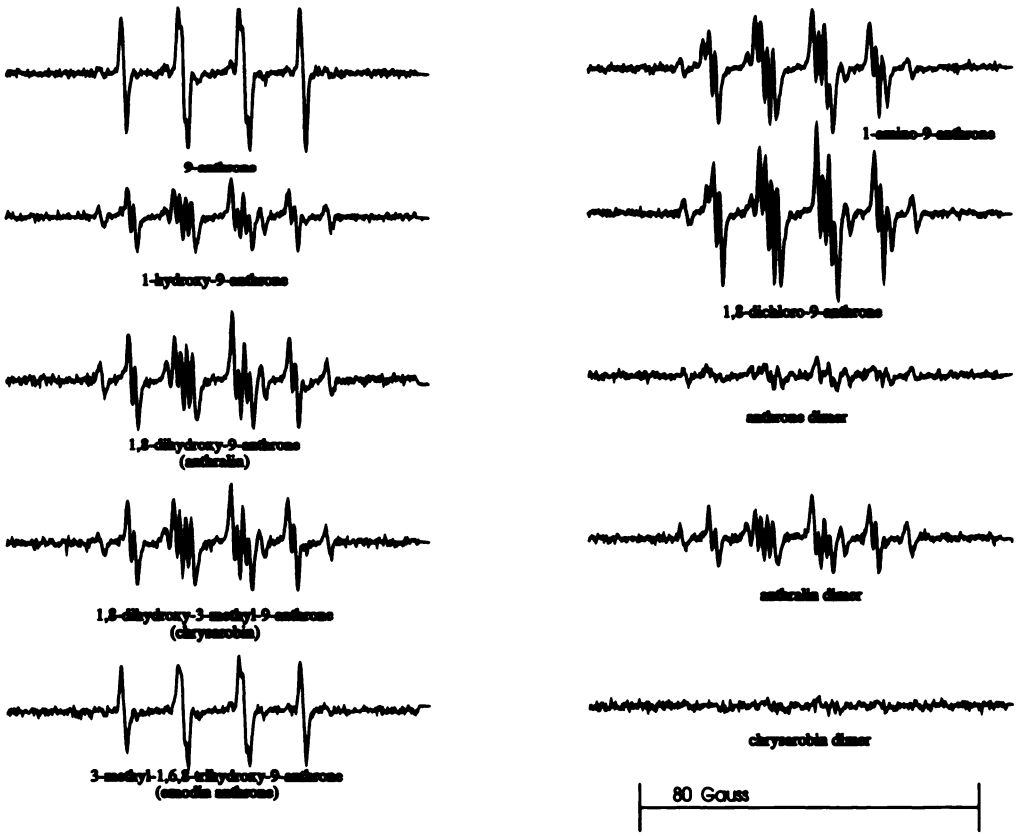
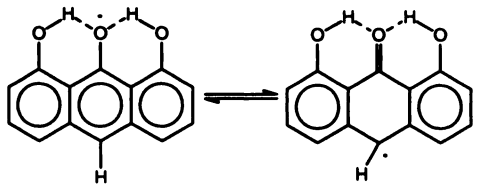
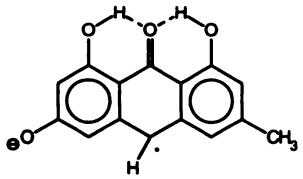


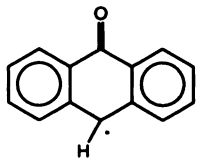
Fig. 5. EPR spectra of DMPO spin adducts produced by autoxidation of various 9-anthrones or 9-anthrone dimers in air-saturated phosphate buffer, pH 7.5, containing 50% Me_2SO. Experimental conditions were identical to those described for Fig. 2.



anthralin radical: stabilized by resonance and hydrogen bonding



Emodin anthrone radical: destabilized by negative charge



9-anthrone radical: no hydrogen bond stabilization

Fig. 6. Structural features of 9-anthrones affecting the stability of 9-anthron-10-yl radicals.

of DMPO is known to decompose enzymatically to form DMPO/·OH (42). As expected, the DMPO/·OH signal was significantly suppressed in the presence of SOD, indicating extracellular trapping of superoxide with subsequent decomposition of the resultant adduct to form DMPO/·OH. However, the broad singlet remained (Fig. 9B). After the anthralin-treated cells were centrifuged and resuspended in fresh buffer containing DMPO, the SOD-sensitive DMPO/·OH signal and the SOD-insensitive singlet could still be observed, indicating that the anthralin was present within the cells. The cells also became yellow, further showing that the hydrophobic anthrones were concentrated within the cells. Significant intracellular trapping of oxygen-centered radicals was not observed. This may be due to inadequate intracellular concentrations of DMPO, the efficient destruction of superoxide by intracellular SOD, or the fast reduction of nitroxide radical spin adducts to EPR silent hydroxylamines or other products (41–43).

Although they have not been well characterized, anthralin brown secondary radicals are thought to be higher oxidation products of anthralin dimer (4, 34). These radicals are detect-

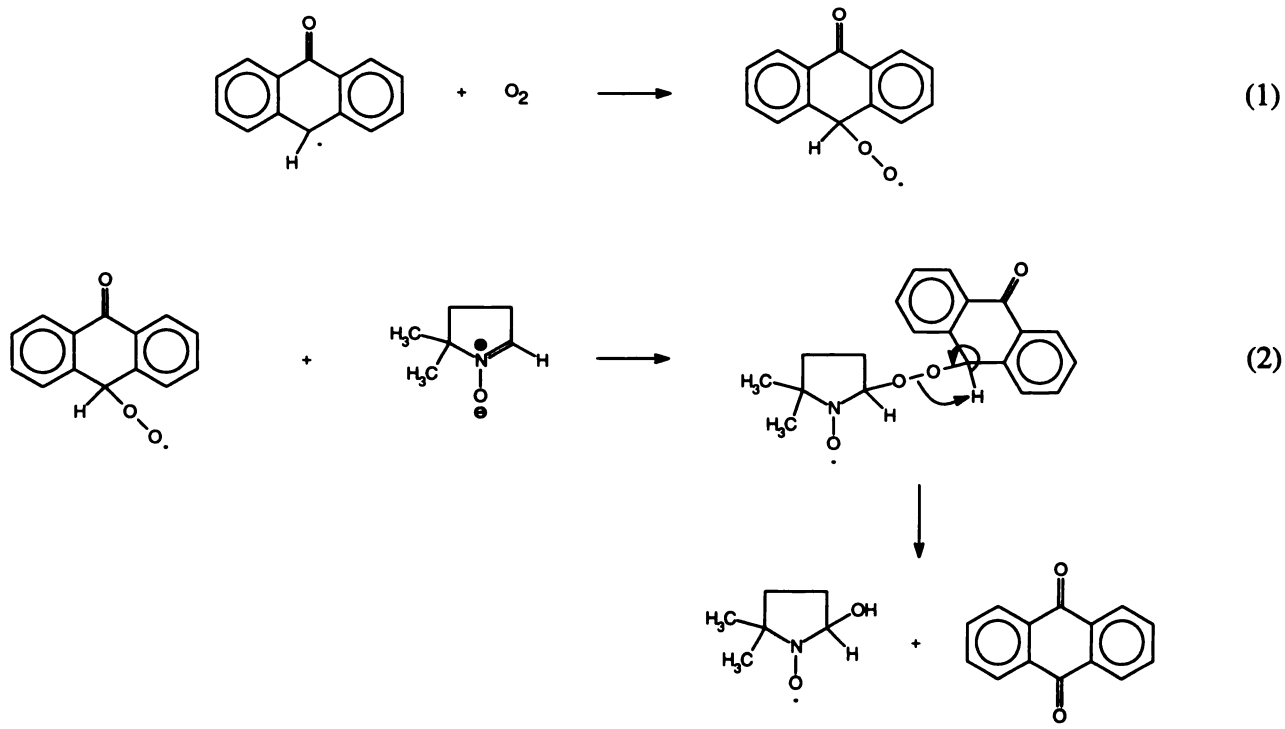


Fig. 7. Proposed mechanism for formation of DMPO/˙OH adducts from DMPO in the presence of 9-anthrones.

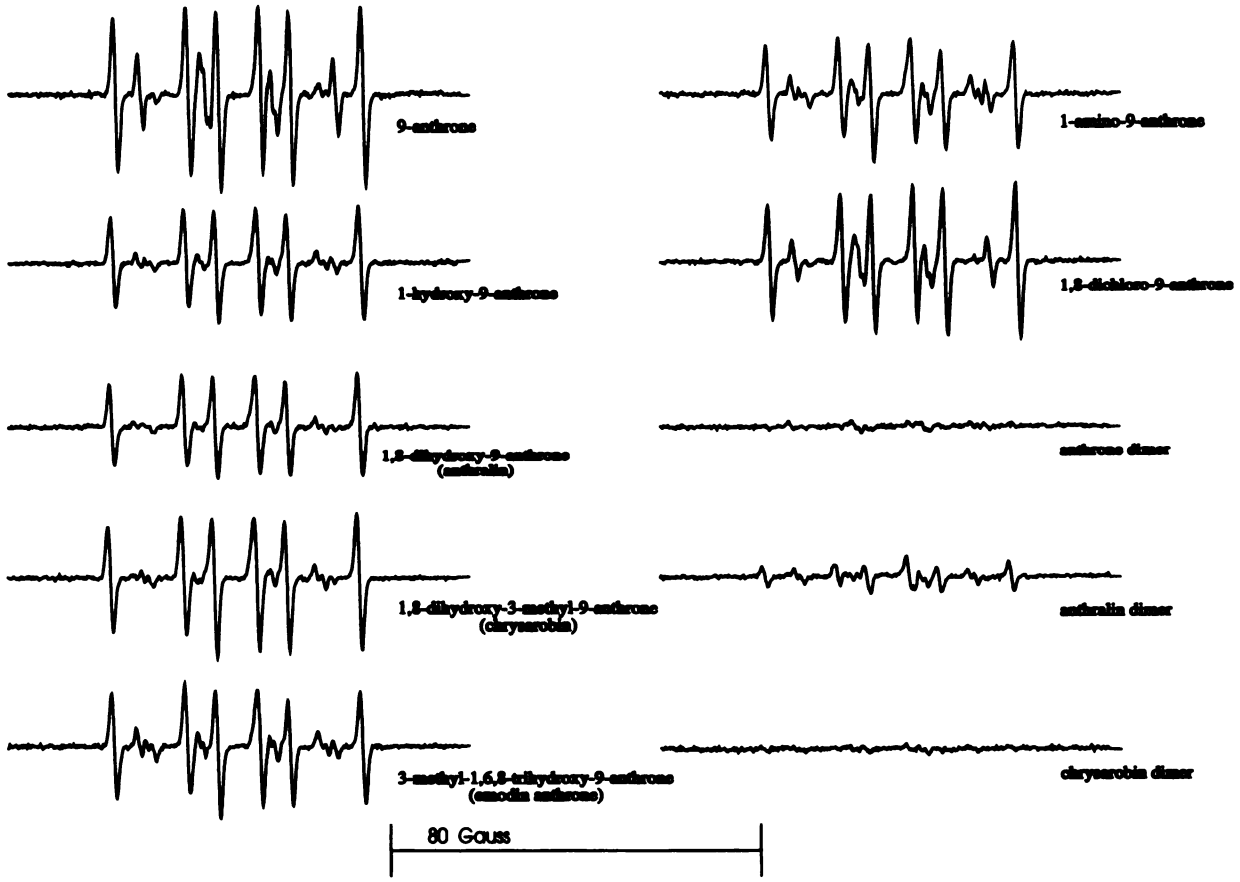


Fig. 8. EPR spectra of DMPO spin adducts produced by autoxidation of various 9-anthrones or 9-anthrone dimers in air-saturated phosphate buffer, pH 7.5, containing 50% Me_2SO and Fe^{3+}/EDTA. Experimental conditions were identical to those described for Fig. 2. EDTA concentration was 1.0 mM and total Fe^{3+} concentration was 10 μM.

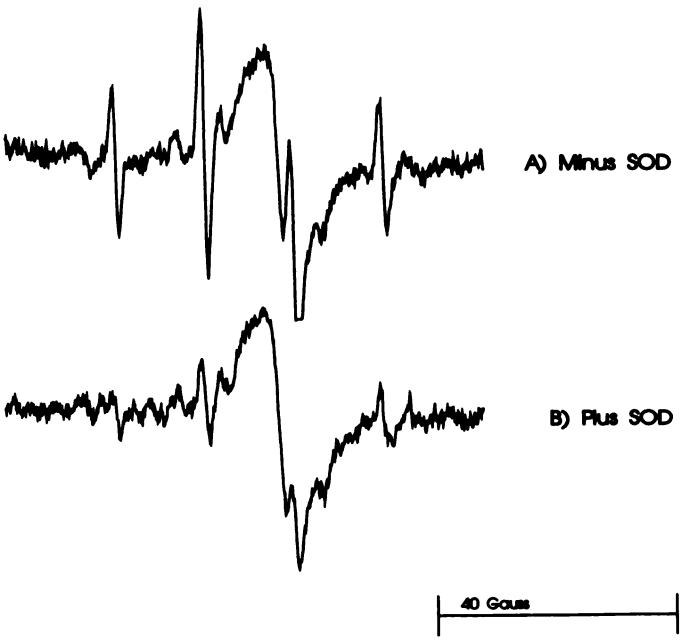


Fig. 9. EPR spectra of keratinocyte suspensions incubated in the presence of anthralin and DMPO. Samples containing 1.16×10^6 cells in 400 μl of buffer (50 mM sodium phosphate, pH 7.5, 150 mM NaCl, 5.0 mM glucose, 1.0 mM DTPA), 20 μl of 2.0 mM anthralin in Me_2SO, 10 μl of DMPO, and 225 units/ml SOD where indicated were incubated for 10 min at room temperature before analysis. Spectra were acquired with 1.0-G modulation amplitude, 327-msec time constant, 5×10^4 gain, and 336-sec scan time. For DMPO/$^{\cdot}$OH, a_N =14.90 G and a_β^H = 14.83 G.

able as a broad singlet signal by direct EPR and have been shown to form in skin treated with anthralin (33, 34), in isolated mitochondria and microsomes (34), and in solutions of peroxidizing lipids (44, 45). We have now found that these radicals are also formed in suspensions of keratinocytes. Therefore, this convenient system was used to generate the radical in the present study.

Because oxidative coupling of aromatic rings in higher oxidation products of 9-anthrone dimers is in some cases known to be catalyzed by visible light (46, 47), we were interested to test the effect of visible light on the formation of the radicals by keratinocytes incubated with anthralin. Further experimentation revealed that brief irradiation with visible light (xenon lamp with window-glass filter) at room temperature and incubation in the dark at 37° were equally efficient at producing the radical signal in keratinocyte suspensions in the presence of anthralin. Heat treatment (95° for 10 min) of the cell suspensions before addition of anthralin only partially prevented the formation of the radical signal. We have also discovered that these radicals form very efficiently during the course of several days in Me_2SO solutions containing anthralin (see below).

When the series of compounds shown in Table 1 were assayed for their abilities to form anthralin brown-like secondary radicals in keratinocyte suspensions, dramatic differences in the ability of the various compounds to form these radicals were found (Fig. 10). Among the three compounds reported in the literature to be effective for the treatment of psoriasis and effective as tumor promoters in mouse skin (Table 1), anthralin was by far the strongest generator of the radical, followed by chrysarobin and 1-hydroxy-9-anthrone.

Emodin anthrone also weakly formed a secondary radical in keratinocytes. Although emodin anthrone possesses what is usually considered to be the minimum structural requirements for antipsoriatic activity (at least one hydroxyl group at the 1- and/or 8-position and at least one hydrogen at the 10-position) (1) and has been presumed to have antipsoriatic activity (29), documentation of its antipsoriatic efficacy could not be found in the literature. Emodin anthrone does not exhibit tumor-promoting activity in mouse skin (17).

Among the compounds reported to be devoid of antipsoriatic activity, 1-amino-9-anthrone produced a weak EPR signal in keratinocytes, whereas 9-anthrone, 9-anthrone dimer, and 1,8-dichloro-9-anthrone did not form radicals in detectable amounts. No information could be found in the literature concerning the tumor-promoting capabilities of 1-amino-9-anthrone, 9-anthrone dimer, or 1,8-dichloro-9-anthrone. 9-Anthrone has been found to be nontumorigenic *in vivo* (11). It is noteworthy that the dimers of the active compounds (anthralin dimer and chrysarobin dimer) formed the radical relatively well, whereas the 9-anthrone dimer, whose corresponding monomer is inactive, did not.

When Me_2SO solutions of the various 9-anthrones or dimers were allowed to age at room temperature and in ambient room light for several days and were then analyzed by direct EPR, the spectra shown in Fig. 11 were obtained. The relative intensities of the signals obtained under these conditions were similar to those obtained in keratinocyte suspensions, except that in this case chrysarobin produced a larger signal than did anthralin and anthralin dimer and chrysarobin dimer produced stronger signals than did their corresponding monomers. The unsubstituted 9-anthrone, its dimer, and 1,8-dichloro-9-anthrone still did not produce detectable signals. These results show that, whereas some 9-anthrones and dimers are capable of slowly forming secondary radicals by an apparent autoxidation process, this process is greatly accelerated in the presence of keratinocytes. The fact that the radical formation in keratinocyte suspensions was only partially prevented by prior heat denaturation of the cells indicates a component of active metabolic oxidation and an autoxidative component.

The differences in the relative signal intensities of the dimers and monomers obtained in keratinocyte suspensions, compared with Me_2SO solutions, may be due to differences in solubility of the test compounds under the respective conditions. Although the compounds used in this study all form true solutions in pure Me_2SO, the dimers are generally less soluble than the monomers. Furthermore, when the compounds are added to aqueous buffer at neutral pH or to tissue culture medium, insoluble aggregates of the compounds often form quickly. This can been seen by filtering the solutions through 0.22-μm membrane filters.

The series of 9-anthrones and dimers were also assayed for their effect on keratinocyte proliferation *in vitro*. The dose-response curves obtained show that, with the exception of anthralin, which inhibited proliferation by 90%, complete inhibition was not attainable (Fig. 12). This is presumably due to poor solubility of the 9-anthrones, as discussed above. In the proliferation experiments solubility problems were further exacerbated because the Me_2SO concentration was necessarily limited to 0.1%, to avoid detachment of the cells from the tissue culture dishes.

The poor aqueous solubility of the test compounds, coupled with their susceptibility to rapid autoxidation once in solution, renders quantitative experiments under these conditions extremely difficult. Nevertheless, the data reveal a qualitative correlation between the ability of the 9-anthrones to form secondary anthralin brown-type radicals in keratinocyte suspensions and their inhibition of keratinocyte proliferation.

Discussion

Superoxide (21–24, 29) and hydroxyl radical (31, 45) formation from anthralin have been extensively documented and

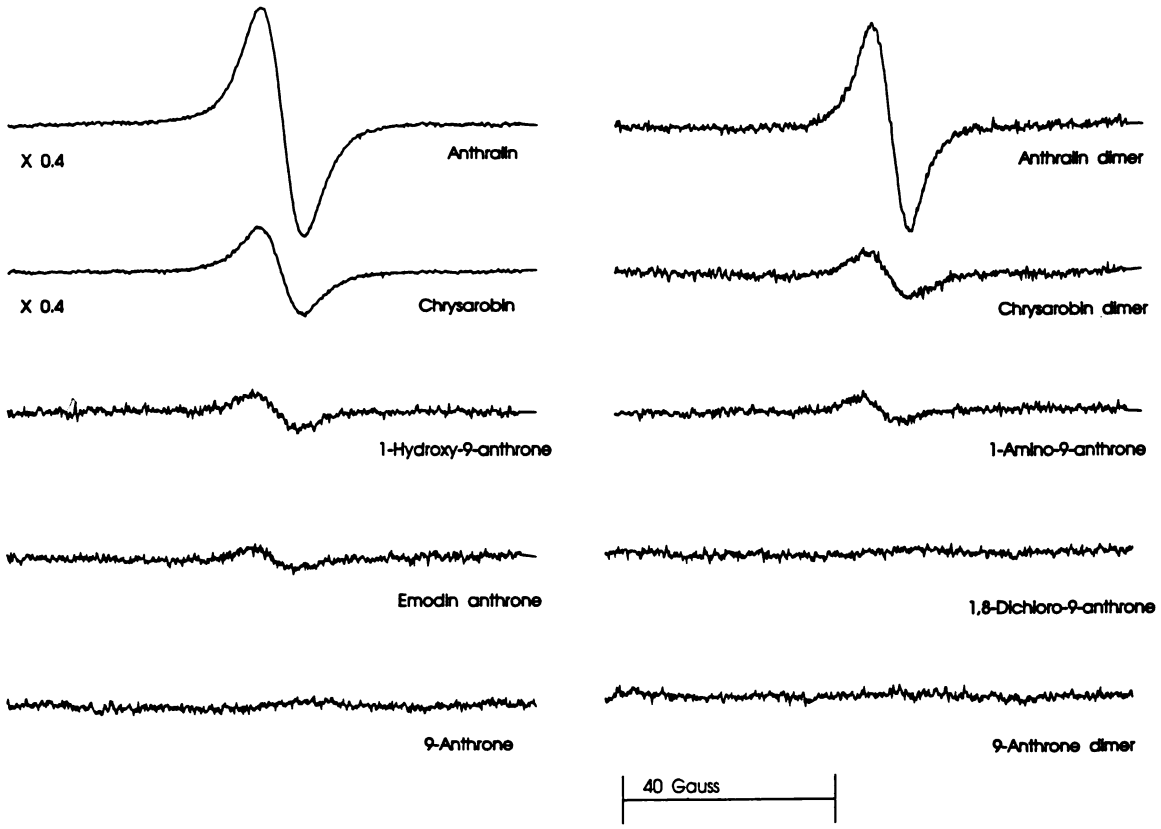


Fig. 10. Direct EPR spectra of mouse keratinocyte suspensions incubated with various 9-anthrone derivatives. Samples containing ~3.0 × 10^6 cells and 100 μl of the indicated 9-anthrone or dimer (2.0 mM in Me_2SO) in 1.0 ml of buffer (50 mM sodium phosphate, pH 7.5, 150 mM NaCl, 5.0 mM glucose, 1.0 mM DTPA) were incubated for 15 min at 37° before analysis. Spectra were acquired at 9.8 GHz with 20-mW microwave power, 100-kHz modulation frequency, 5.73-G modulation amplitude, 327-msec time constant, 2.0 × 10^4 gain, and 327-sec scan time. Anthralin and chrysarobin are shown at 40% of full scale.

have been hypothesized to play important roles in mediating its biological effects. In the present study we have confirmed the formation of both superoxide and hydroxyl radical from anthralin and other antipsoriatic and tumor-promoting 9-anthrones in aqueous buffer/Me_2SO solutions. The mechanism of hydroxyl radical formation was shown to be via dismutation of superoxide to form hydrogen peroxide, followed by superoxide- or anthralin-dependent Fenton reaction. Fe^{3+}/EDTA, but not Fe^{3+}/DTPA, complexes could be efficiently reduced by superoxide or anthralin. This is consistent with the reactivity of superoxide with Fe^{3+} complexes previously reported by other workers (Ref. 48 and references cited therein).

It was further shown that several 9-anthrones that have been found previously by other workers to be devoid of tumor-promoting or antipsoriatic activities also produce reactive oxygen species, including the hydroxyl radical, as easily as do the active compounds (see Table 1 for literature references to known biological activities). Additionally, evidence was obtained to suggest that, for 9-anthrones that cannot stabilize 10-yl radicals well, reaction with molecular oxygen to form the corresponding 10-peroxyl radical also readily occurs. In these cases, reduction of subsequently formed 9-anthron-10-hydroperoxides by Fe^{2+} (or perhaps metabolically in living systems) might also result in the formation of hydroxyl radical.

We could not use the spin-trapping technique to demonstrate significant generation of reactive oxygen radicals within cells. However, because all of the compounds tested are hydrophobic and readily enter cells, and because metabolism is not required for reactive oxygen species production, specificity for transport or metabolism may not be important factors when these results are extrapolated to cellular systems.

The data obtained in the present study reveal that the ability of 9-anthrones to generate oxygen-centered radicals does not correlate with the reported tumor-promoting or antipsoriatic activities of these compounds. A similar lack of correlation between redox-cycling ability and biological activity has been noted for redox-cycling quinones (26). Thus, although the evidence of a role for oxidants in tumor promotion (49) is very strong, it seems likely that for the 9-anthrones additional factors are also involved. Because the inactive (with respect to antipsoriatic or tumor-promoting activity) compounds or their anthraquinone oxidation products all generate active oxygen species and are known to be powerful skin irritants, the data are consistent with a role for these species in the inflammatory response. This postulate is supported by reports that antioxidants effectively prevent anthralin-induced skin irritation (8, 50).

In contrast to their ability to produce active oxygen species, dramatic differences in the ability of the series of compounds to form an anthralin brown-like radical in keratinocyte suspensions or in Me_2SO solutions were revealed. The 1- and/or 8-hydroxy-substituted compounds and the 1-amino-substituted compound were found to produce these radicals. Hydroxy substitution at both the 1- and 8-positions provides maximum radical-producing ability, whereas further substitution at both the 3- and 6-positions leads to diminished activity. The ability of the 9-anthrones and dimers to produce secondary radicals in keratinocytes also correlates with their antiproliferative activities *in vitro*. In this regard, the results obtained with the 9-anthrone dimers are especially noteworthy, because anthralin dimer has been reported to be devoid of both antipsoriatic (25) and tumor-promoting (10) activity *in vivo*. This is discussed in more detail below.

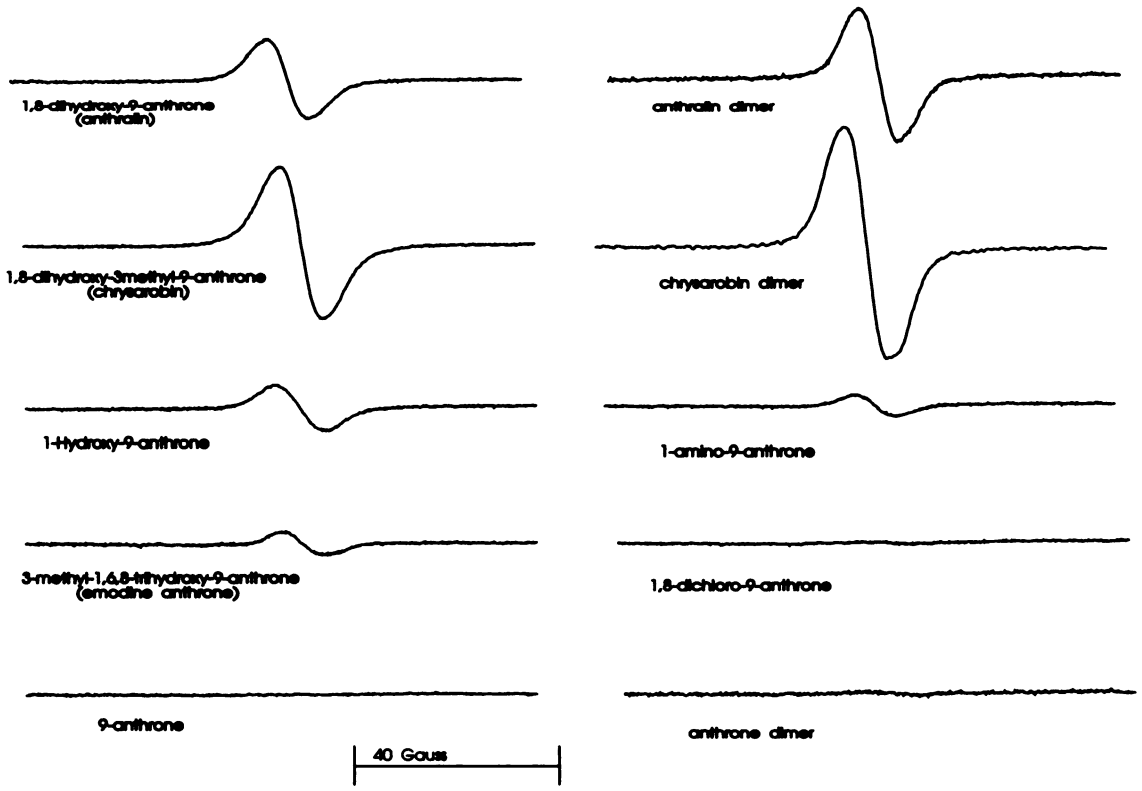


Fig. 11. Direct EPR spectra of Me_2SO solutions of various 9-anthrones or 9-anthrone dimers. Air-saturated Me_2SO solutions of the indicated compounds (1.0 mM) were aged in capped clear borosilicate glass vials at room temperature with ambient room lighting conditions for 3 days and were then diluted 10-fold into aqueous carbonate/bicarbonate buffer, pH 10.0, before analysis. Spectra were acquired with 4.125-G modulation amplitude, 250-msec time constant, 5×10^3 gain, and 4-min scan time.

The relative order of secondary radical-producing ability of 9-anthrones was found to be anthralin > chrysarobin ≫ 1-hydroxy-9-anthrone > emodin anthrone = 1-amino-9-anthrone. Anthralin, chrysarobin, and 1-hydroxy-9-anthrone have all been reported to have antipsoriatic efficacy (26). However, no quantitative information regarding their relative potencies was reported. Documentation of the antipsoriatic efficacy for emodin anthrone could not be found in the literature. 1-Amino-9-anthrone, which formed the radical only very weakly, and 9-anthrone and 1,8-dichloro-9-anthrone, which did not form the radical at all, were found to have no efficacy against psoriasis (26).

With regard to tumor promotion, anthralin and chrysarobin were reportedly similar in activity (17), whereas 1-hydroxy-9-anthrone was significantly less potent than anthralin (11). Emodin anthrone (17), which formed a secondary radical only very weakly, and 9-anthrone (7), which did not form a secondary radical, were found to be nontumorigenic. No information was found in the literature concerning the tumor-promoting or antipsoriatic effects of the remaining test compounds. Thus, with the notable exception of anthralin dimer, the secondary radical-forming abilities and *in vitro* antiproliferative activities correlate reasonably well with the known *in vivo* activities of these compounds. Although weak secondary radical-forming ability correlated well with weak antiproliferative activity *in vitro*, secondary radical-forming ability less than that of 1-hydroxy-9-anthrone is apparently insufficient to produce measurable effects *in vivo*.

Anthralin brown secondary radicals are thought to be higher oxidation products of anthralin dimer (4, 34). However, because of their remarkable stability, an important role for these secondary radicals in mediating the biological effects of 9-anthrones has been considered by some to be unlikely (34). Consistent with this notion is the fact that anthralin dimer has no antisporiatic activity (24) and is nontumorigenic *in vivo* (10). Nevertheless, the importance of secondary radical formation with respect to the biological activities of 9-anthrones has been suggested previously by Ducret and co-workers (44, 45). They reported an anthralin-derived radical, similar to that found in anthralin-treated skin and that detected in anthralin-treated keratinocytes in the present report, which was formed over the course of several months in solutions of peroxidizing chicken fat lipids containing anthralin (44). Because the secondary radical formed concomitantly with the disappearance of anthralin dimer, those authors suggested the dimer as an intermediate (44). In a later study they noted a correlation between secondary radical formation and inhibition of mitochondrial respiration in keratinocytes, although no data regarding the antirespiratory activity of the dimer were reported (45).

As mentioned above, a problem with any hypothesis in which the dimer is an important intermediate involved in secondary radical formation and biological responses lies in the fact that the dimer has no apparent clinical activity (10, 25). The dimer has, however, been reported to be about as effective as anthralin for inhibition of keratinocyte proliferation *in vitro* (20, 51). The *in vitro* experiments presented here confirm the antiproliferative and secondary radical-producing effect of anthralin dimer and further reveal a trend that shows that the dimers of the active 9-anthrones are also active, whereas the dimer of an inactive 9-anthrone is not.

The apparent inconsistencies between the *in vivo* and *in vitro* activities of the dimers may be explained by postulating the dimer as an intermediate that is too reactive to survive pene-

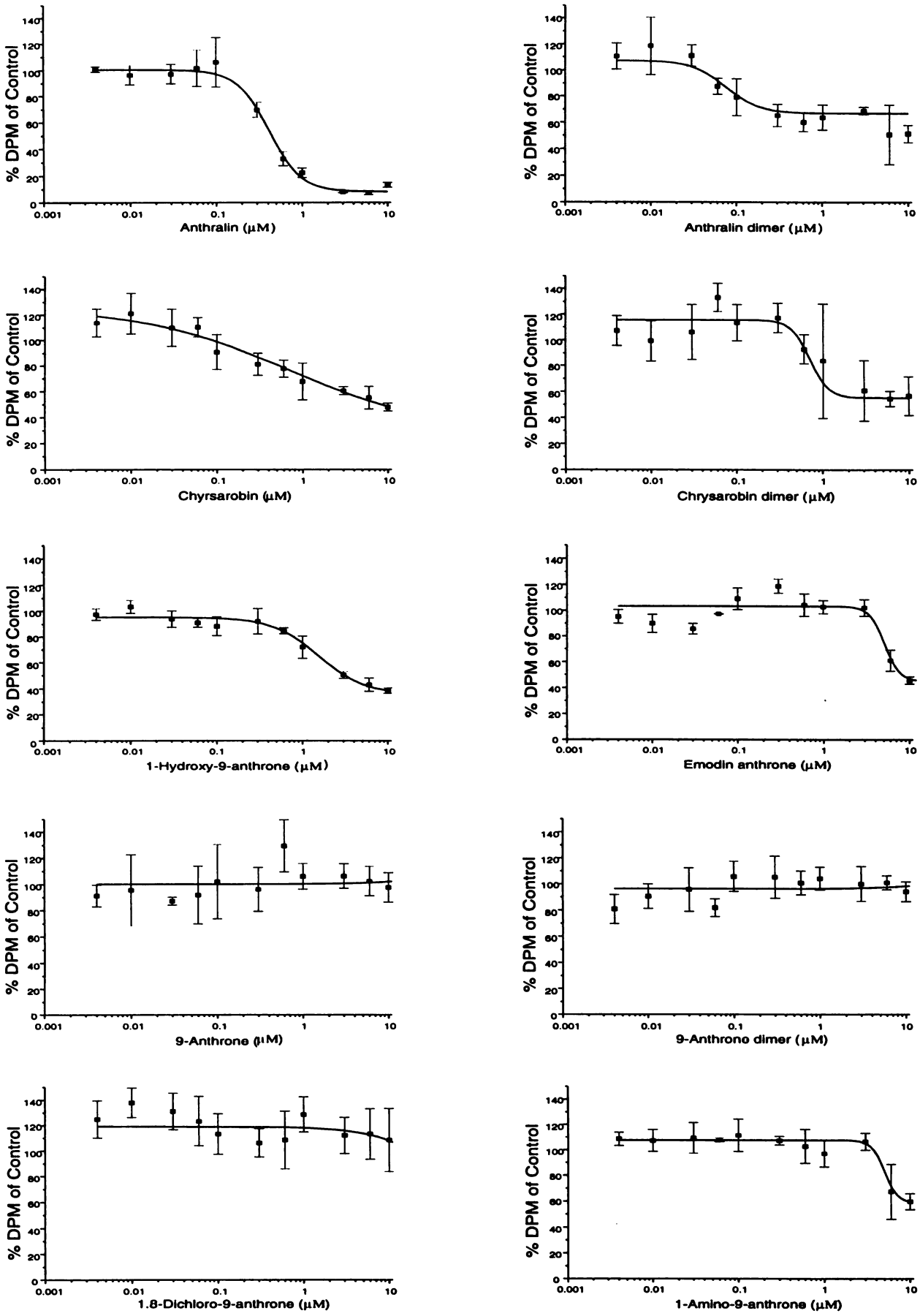


Fig. 12. Dose-response curves for the inhibition of keratinocyte proliferation by various 9-anthrones and 9-anthrone dimers. Proliferation was measured as the amount of [^{3}H]thymidine incorporated into keratinocyte DNA. The experimental protocol is described in Experimental Procedures. Sigmoidal curves or apparent linear fits were applied to the data by computer (Origin Technical Graphics and Data Analysis in Windows; Microcal Software Inc., Northhampton, MA). Data points are presented as the mean ± standard deviation of the following number of experiments: chrysarobin, five; 9-anthrone, six; all others, three.

tration of the skin (resulting in the formation of the anthralin brown products and staining), but that must be formed beneath the protective barrier of the stratum corneum in, or in close proximity to, live keratinocytes in order to reach its critical biological target. As pointed out by Fuchs and Packer (34), the stability of anthralin brown radicals makes their role in mediating biological effects questionable. However, even if these radicals are themselves not directly involved in producing biological effects, the ability to form them appears to be a useful indicator of an inherent reactivity of the parent compounds that is important for their bioactivity.

It has recently been reported that antioxidants block the tumor-promoting effects of the 9-anthrones (18). Although it is reasonable to interpret these results as indicating an important role for oxygen radicals, the results presented in this paper indicate that the involvement of other types of free radical species must also be considered. In this regard, it is interesting to note that EPR experiments have shown that antioxidants also block the formation of anthralin brown radicals in skin.[4]

Only the tumor-promoting and antipsoriatic compounds or their dimers produced significant secondary radicals in keratinocyte suspensions. These same compounds also inhibited keratinocyte proliferation *in vitro*, indicating a cytotoxic effect. This is in contrast to the effects of hydrogen peroxide or the phorbol ester tumor promoters, both of which increase DNA synthesis and proliferation of cells in culture (52, 53). Thus, the *in vivo* hyperplasia induced by 9-anthrones may be the result of a regenerative response following cytotoxicity. This has been suggested previously (16) to account for the tumor-promoting effects of the 9-anthrones and has also been suggested to be the mechanism of action of other tumor promoters, such as thapsigargin (53). Strong evidence exists to suggest that chronic regenerative hyperplasia caused by any source, including mechanical wounding of the skin, can result in tumor promotion (54).

In summary, the results presented in this study indicate that the ability of 9-anthrones to generate reactive oxygen radicals is not sufficient to produce antiproliferative activity in keratinocytes *in vitro*. Generation of reactive oxygen species by 9-anthrones does not correlate with their known tumor-promoting and antipsoriatic capabilities but is consistent with a role for these species in mediating 9-anthrone-induced inflammation and irritation. Although the evidence of a role for reactive oxygen radicals in tumor promotion is compelling, the ability to generate these species may, by itself, not be sufficient to confer tumor-promoting activity on the 9-anthrones. Our results also provide a strong indication that the ability to form an anthralin brown-type secondary radical is an important determinant of 9-anthrone antiproliferative activity in keratinocytes, and possibly of antipsoriatic and tumor-promoting activity as well. Whether the radical itself is the active species or whether the ability to form the radical is simply a correlated phenomenon is not clear. Only the hydroxy-substituted 9-anthrones or their dimers possessed significant secondary radical-forming and antiproliferative activity.

Knowledge of the chemical structure of the secondary radicals would undoubtedly shed important light upon the chemistry involved in their formation and thus, perhaps, upon the chemistry involved in mediating the biological activities of the 9-anthrones. The currently available information suggests the possible importance of reactive dimeric intermediates. Unfortunately, little information regarding the structure of the secondary radicals is revealed by the EPR spectra, because the broad singlet signals they produce lack any resolvable hyperfine structure. Therefore, we are currently attempting to determine the structures of 9-anthrone oxidation products by other methods.

[4] H. Utsumi, Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan, personal communication.

Acknowledgments

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