The Autoxidation of 6-Hydroxybenzo[*a*]pyrene and 6-Oxobenzo[*a*]pyrene Radical, Reactive Metabolites of Benzo[*a*]pyrene†

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ABSTRACT: A labile metabolite of the carcinogen benzo[*a*]pyrene, 6-hydroxybenzo[*a*]pyrene, is autoxidized in aqueous buffer–ethanol solutions to produce the three stable 6,12- 1,6-, and 3,6-benzo[*a*]pyrene diones and a small amount of an unidentified paramagnetic, violet-colored material. In this reaction, oxygen is reduced to hydrogen peroxide and probably to other reactive reduced oxygen species as well. A free radical derived from 6-hydroxybenzo[*a*]pyrene by one-electron oxidation, 6-oxobenzo[*a*]pyrene radical, is most likely an obligatory intermediate in this autoxidation as indicated by its kinetics of formation and decay. In addition, this free radical can be produced quantitatively in benzene by oxidation of 6-OH-B[*a*]P with aqueous $K_3Fe(CN)_6$ and isolated in the absence of oxygen. Like 6-hydroxybenzo[*a*]pyrene, this isolated free radical is autoxidized in aqueous buffer–ethanol solutions and yields identical products. 6-Hydroxybenzo[*a*]pyrene is also oxidized rapidly in rat liver homogenate to the three benzo[*a*]pyrene diones, indicating that it is a labile precursor of these often-found products of benzo[*a*]pyrene metabolism. The production of reactive hydrocarbon and reduced oxygen intermediates can explain the results of previous studies which demonstrated that 6-hydroxybenzo[*a*]pyrene binds covalently to, and causes strand breakage in, nucleic acid. This reactivity of 6-hydroxybenzo[*a*]pyrene may also be related to its high toxicity toward cells in culture and its ability to morphologically transform Syrian hamster embryo fibroblasts.

During the last 20 years considerable evidence has been accumulating in support of the hypothesis that cellular metabolism is a prerequisite for the carcinogenic activity of many chemical carcinogens, particularly the polycyclic aromatic hydrocarbons. Among these is the potent environmental carcinogen, benzo[*a*]pyrene, B[*a*]P,[1] which is metabolized, to many identified, stable products by hepatic microsomes (Holder et al., 1974; Selkirk et al., 1974) as well as by other in vitro and in vivo systems.

The 6 position of B[*a*]P is chemically more reactive than the other sites on this hydrocarbon, as indicated by studies of its anodic oxidation (Jeftic and Adams, 1970), chemical substitution (Fieser and Hershberg, 1939), and hydrogen exchange (Cavalieri and Calvin, 1971). In early studies of B[*a*]P metabolism, a few workers detected a monohydroxylated metabolic product, 6-hydroxybenzo[*a*]pyrene, 6-OH-B[*a*]P (Falk et al., 1962; Pihar and Spaleny, 1956; Casu et al., 1951). More recently, Nagata et al. (1974) demonstrated that this transient metabolite can be conveniently detected in the metabolism of B[*a*]P in rat liver homogenate via its phenoxy radical, 6-oxo-B[*a*]P radical. This observation has been confirmed and extended by our laboratory as reported in the accompanying paper (Lesko et al., 1975). Our biochemical studies and electron spin resonance (ESR) measurements show that the 6-OH-B[*a*]P pathway accounts for a significant proportion of the total metabolism of B[*a*]P in rat hepatic homogenate. In contrast, conventional chromatographic analysis of B[*a*]P metabolic products most often has not revealed the presence of this labile compound, a point which is discussed in more detail in the accompanying paper (Lesko et al., 1975).

In the present study, the process and the products of the autoxidation of 6-OH-B[*a*]P have been examined. The 6-oxo-B[*a*]P radical was found to be an obligatory intermediate leading to the formation of three B[*a*]P diones (1,6-, 3,6-, and 6,12-B[*a*]P dione) which accounts for greater than 90% of the oxidation products. These B[*a*]P diones usually have been recognized to constitute a substantial portion of the B[*a*]P metabolites (Selkirk et al., 1974; Holder et al., 1974). The consumption of molecular oxygen in the autoxidation of B[*a*]P has been directly measured. The molecular oxygen is reduced to reactive species as demonstrated by the formation of hydrogen peroxide during the reaction.

It should be noted that in ethanol–phosphate buffer (1:1, v/v) solution, 6-OH-B[*a*]P spontaneously reacts with nucleic acid forming covalent bonds and causing strand breakage (Ts'o et al., 1974). Some of the reactive species in the autoxidation of 6-OH-B[*a*]P may participate in these important chemical reactions with nucleic acids. More recently, we have reported (Schechtman et al., 1974) that 6-OH-B[*a*]P is highly toxic to Syrian hamster embryo fibroblasts and that morphological transformation has been observed in clones surviving this treatment.

Experimental Section

Materials

Benzo[*a*]pyrene and 3,3-dimethoxybenzidine (*o*-dianisidine) were purchased from Aldrich Co., Milwaukee, Wis.; horseradish peroxidase (EC 1.11.1.7) was purchased from

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[1] Abbreviations used are: B[*a*]P, benzo[*a*]pyrene; 6-OH-B[*a*]P, 6-hydroxybenzo[*a*]pyrene; B[*a*]P-dione, benzo[*a*]pyrene dione; 6-oxo-B[*a*]P, 6-oxobenzo[*a*]pyrene.

Worthington Corp., Freehold, N.J.; rat liver homogenate was prepared from adult Sprague-Dawley or ACI rat livers as described in the accompanying paper (Lesko et al., 1975).

6-Hydroxybenzo[a]pyrene. 6-Acetoxybenzo[a]pyrene was prepared from benzo[a]pyrene according to the procedure of Fieser and Hershberg (1938). It was purified further by elution from a silica gel column with benzene followed by recrystallization from benzene, mp 209–209.5°. This 6-acetoxybenzo[a]pyrene was added to an ether slurry of lithium aluminum hydride (5–10-fold excess). The mixture was stirred under an atmosphere of N_2 in the dark for 4 hr at room temperature. Careful neutralization with 1 *M* HCl was carried out at 0° as rapidly as conditions permitted. Care was taken not to allow the mixture to become acidic. Rapid ether extraction and drying of the ether solution (anhydrous Na_2SO_4) was followed by flash evaporation of the solvent (all at 0°). The yellow solid was sublimed slowly in vacuo at 150–160° giving a yellow, microcrystalline product: mp 180° dec; λ_{max}(benzene) 388 nm (ϵ 20,300); mass spectrum 268 (molecular ion), 252 (M − O), 240 (M − CO), and 239 (M − CHO). The compound is not stable in solution; purification by solution methods did not give satisfactory results. Air exposure gradually turns the solid compound to dark yellow-green in color and results in increasing amounts of B[a]P dione impurities. It should be stored as a solid under N_2, or better, under vacuum desiccation.

Apparatus

Ultraviolet and visible absorption spectra were measured on Cary Models 14 and 15 recording spectrophotometers. Fluorescence spectra were measured on an Aminco-Bowman spectrofluorophotometer. Electron spin resonance was measured on a JEOL Model JES-ME-1X ESR spectrometer. The quantitation of the ESR measurement is based on double integration of the derivative signal and comparison to a known MnO concentration standard. Oxygen tension was measured on an oxygen electrode from Yellow Springs Instruments and recorded automatically on a Heathkit recorder.

Procedures

Autoxidation of 6-Hydroxybenzo[a]pyrene. 6-Hydroxybenzo[a]pyrene was dissolved in 95% ethanol and an equal volume of 0.01 *M* sodium phosphate buffer (pH 7.0) was added to make the solution 1:1 (v/v) 95% ethanol–buffer. Concentrations of 6-OH-B[a]P in solution were 5×10^{-4} *M* unless otherwise stated. The solution was allowed to stand at room temperature (22°) in the dark for at least 2 days. Some of the ethanol was evaporated and all of the colored material was extracted into benzene. This extract was applied to an alumina (activity grade III) column and first eluted with distilled benzene. Three B[a]P diones were collected which accounted for 90–92% of the theoretical yield. This determination of the B[a]P diones was made by absorption spectroscopy using the extinction coefficient data obtained from the highly purified products shown in Figure 2. When the product mixture was not eluted quickly from the column, the yields were lower. Analysis of absorption spectra before and after chromatography suggests that the B[a]P diones were degraded by the exposure to alumina, and the degradation products could not be eluted from the column.

The first compound to be eluted from the column was the 6,12-benzo[a]pyrene dione which was purified further by thick layer alumina chromatography (benzene–ETOH, 99:1) and by recrystallization from toluene. The product forms rust-colored, fine needles: mp 320–321°; mass spectrum 282 (molecular ion). Next to be eluted from the column was 1,6-benzo[a]pyrene dione. The first half of this fraction was purified further by thick layer alumina chromatography (benzene–EtOH, 99:1) and by recrystallization from toluene. The product forms orange plates: mp 287–288°; mass spectrum 282 (molecular ion). The third compound eluted was 3,6-benzo[a]pyrene dione. Complete separation between the 1,6 and 3,6 diones was not easily obtainable as a small amount of 1,6 dione tailed extensively into the 3,6 dione fraction. The 3,6 dione was purified by successive (five times) thick layer chromatography (benzene–EtOH, 99:1) and by recrystallization from toluene. The product forms red, fine needles: mp 288–289°; mass spectrum 282 (molecular ion). Elution was continued with benzene–ethanol (98:2) and a small amount of a violet-colored (absorption λ_{max} 527, 568, and 602 nm in benzene), orange fluorescing ($\lambda_{max\ emission}$ 577 nm, in benzene) material was obtained. This material also displayed an ESR spectrum (singlet, g = 2.005, line width 7 G, in benzene). No hyperfine structure of this ESR signal was observed even by degassing the solution.

Oxygen Uptake. 6-Hydroxybenzo[a]pyrene was dissolved in 95% ethanol and an equal volume of 0.01 *M* sodium phosphate (pH 7.0) was added to make the solution 1:1 (v/v) 95% ethanol–buffer. This solution was quickly transferred to completely fill a closed, thermostated chamber (3 ml) fitted with an oxygen electrode. The electrode potential was measured by a potentiometer and recorded automatically.

Hydrogen Peroxide Assay. The assay adopted is a modification of the method of Gregory (1966). This method was developed by C. S. Worthington and J. D. Teller for assay of horseradish peroxidase as described in the Worthington catalogues. Two identical aliquots (1.0 ml each) of a 6-OH-B[a]P solution in 1:1 (v/v) 95% ethanol–0.01 *M* sodium phosphate (pH 7.0) were prepared. To one portion was added 50 μl of 1% dianisidine in methanol and 50 μl of horseradish peroxidase in water (1 mg/ml). To the reference portion was added 50 μl of 1% dianisidine in methanol and 50 μl of water. These two portions were individually mixed and quickly measured for difference in absorptivity at 600 nm. It was necessary to use this wavelength rather than the absorbance maximum (at 460 nm) of the oxidized form of *o*-dianisidine because of the high extinction of 6-OH-B[a]P and the B[a]P dione products in this region. The extinction coefficient of the oxidized form at 600 nm is 1.38×10^3. It was determined in the presence of 6-OH-B[a]P by the addition of known amounts hydrogen peroxide to solutions containing *o*-dianisidine and peroxidase.

Preparation of 6-Oxo-B[a]P Radical. 6-OH-B[a]P was dissolved in N_2-sparged benzene at the concentration of 10^{-3}–10^{-4} *M* in a glove bag filled with nitrogen. An equal volume of an N_2-sparged, aqueous solution of $K_3Fe(CN)_6$ at 10^{-2} *M* was added to the benzene solution. The mixture was shaken and the yellow benzene layer turned almost immediately to a yellowish green color, indicating the conversion to the 6-oxo-B[a]P radical. The $K_3Fe(CN)_6$ concentration is needed in large excess. The benzene layer was then separated from the aqueous solution and was dried by anhydrous Na_2SO_4. The 6-oxo-B[a]P radical in dried benzene is stable under N_2.

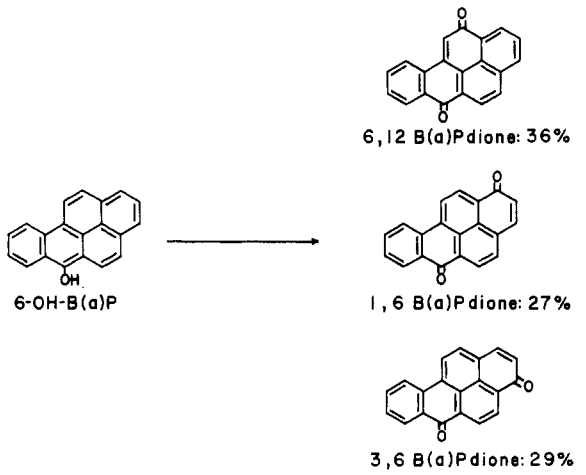

FIGURE 1: The three major products and their yields obtained from the autoxidation of 6-hydroxybenzo[*a*]pyrene in 1:1 (v/v) 95% ethanol–0.01 *M* sodium phosphate (pH 7.0).

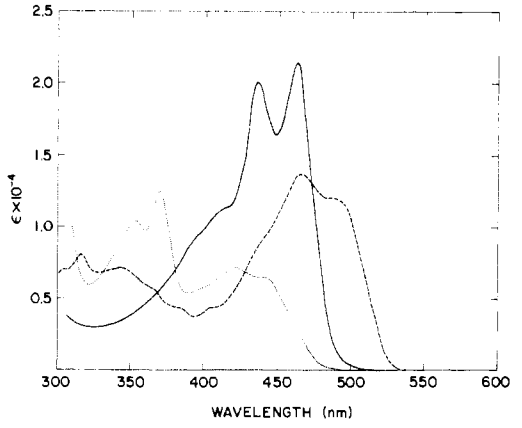

FIGURE 2: Absorption spectra and extinction coefficients of the B[*a*]P diones in benzene, 6,12-B[*a*]P dione (. . .), 1,6-B[*a*]P dione (—), 3,6-B[*a*]P dione (- - -).

Results

Autoxidation of 6-Hydroxybenzo[a]pyrene. The instability of 6-OH-B[*a*]P upon exposure to air was first noticed upon purification of the compound during the synthesis, which is described in the Experimental Section. In air, darkening of the surface of the freshly sublimed compound can be noticed within an hour, and the autoxidation process is accelerated when the compound is dissolved in polar solvents such as Me_2SO, ethanol, and acetone.

The major products of autoxidation are 6,12-benzo[*a*]pyrene dione (6,12-B[*a*]P dione), 1,6-benzo[*a*]pyrene dione (1,6-B[*a*]P dione), and 3,6-benzo[*a*]pyrene dione (3,6-B[*a*]P dione). Figure 1 shows the percent yields of these diones obtianed when 6-OH-B[*a*]P was incubated for 2 days at room temperature in 1:1 (v/v) 95% ethanol–0.01 *M* sodium phosphate (pH 7.0). These products were separated on alumina by column and thick layer chromatography (see Procedures). The absorption spectra as well as the extinction coefficients of these pure products are shown in Figure 2, and these spectra proved to be useful for the quantitative determination of the B[*a*]P dione products. Because of their high extinction coefficients, routine analyses of products could be made of the reaction starting with 1 μmol or less of 6-OH-B[*a*]P. At this level, over 90% of the theoretical yield was accounted for as the three B[*a*]P diones.

In addition, a small amount of a violet, intensely orange-fluorescing material was eluted from the alumina column after the diones. The fluorescence spectrum of this material is shown in Figure 3. This material also possesses an ESR signal which is a broad singlet (g = 2.005, linewidth 7 G, in benzene). Apparently, this is the same free radical signal which persists in the mixture of 6-OH-B[*a*]P oxidation products long after the B[*a*]P diones have formed. Attempts to obtain hyperfine structure by degassing were unsuccessful. The signal does not decay over a period of weeks. Quantitative measurement of this ESR signal by double integration and comparison with a known MnO standard indicated that this radical is produced in 1–3% yield in the autoxidation.

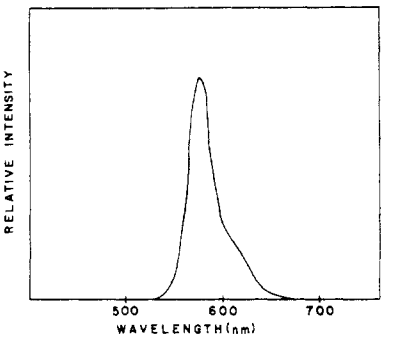

FIGURE 3: Fluorescence emission spectrum of the violet-colored autoxidation product of 6-hydroxybenzo[*a*]pyrene in benzene, $\lambda_{excitation}$ 400 nm.

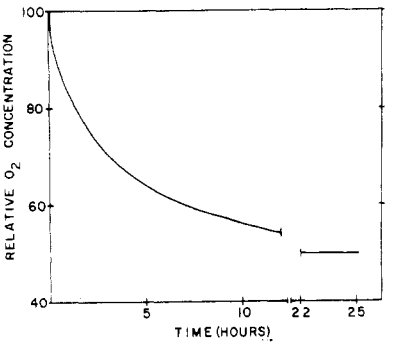

FIGURE 4: The consumption of oxygen during the oxidation of 6-hydroxybenzo[*a*]pyrene (0.52 m*M*) in 1:1 (v/v) 95% ethanol–0.01 *M* sodium phosphate (pH 7.0) at 22° as measured with an oxygen electrode.

The four-electron oxidation of 6-OH-B[*a*]P to the B[*a*]P diones consumes oxygen which is dissolved in solution. The kinetics of this consumption are presented in Figure 4. In this experiment, the oxygen electrode measurements were made in a closed system containing the 6-OH-B[*a*]P dissolved in 1:1 ethanol–phosphate buffer solution. The molecular oxygen consumed in this oxidation is reduced and results, in part, in the production of hydrogen peroxide. Figure 5 shows the presence of hydrogen peroxide during the 6-OH-B[*a*]P oxidation. The rather complex kinetics of the formation and decay of hydrogen peroxide suggests that its involvement in this process may be complex, a notion that will be further discussed.

Presence of the 6-Oxobenzo[a]pyrene Radical during the Autoxidation. During the autoxidation of 6-OH-B[*a*]P, a free radical is produced whose characteristic ESR spectrum is shown in Figure 6. The symmetry of the signal suggests that the hyperfine structure results mainly from one free radical species. In aqueous–ethanol (Figure 6) or benzene solution (Lesko et al., 1975) the radical produces five ESR lines at high modulation (>1 G) indicating that

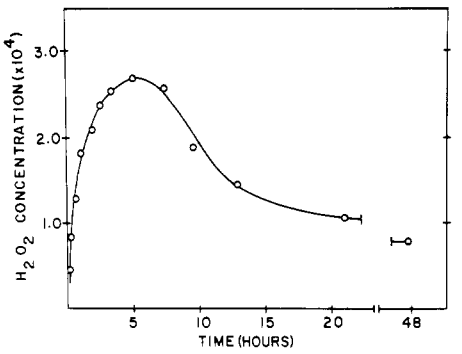

FIGURE 5: The concentration level of hydrogen peroxide during the autoxidation of 6-hydroxybenzo[a]pyrene (0.52 mM) in 1:1 (v/v) 95% ethanol-0.01 M sodium phosphate (pH 7.0) at 22°.

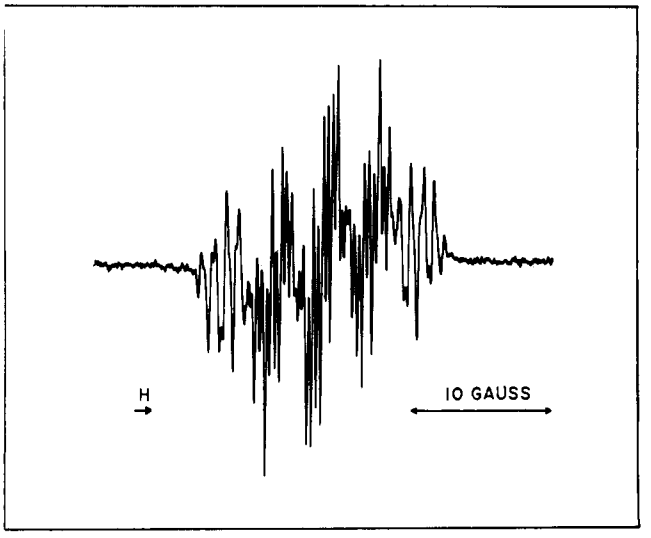

FIGURE 6: The ESR spectrum of the 6-oxobenzo[a]pyrene radical in 1:1 (v/v) 95% ethanol-0.01 M sodium phosphate (pH 7.0), modulation amplitude 0.10 G, power 6 mW, g = 2.0038.

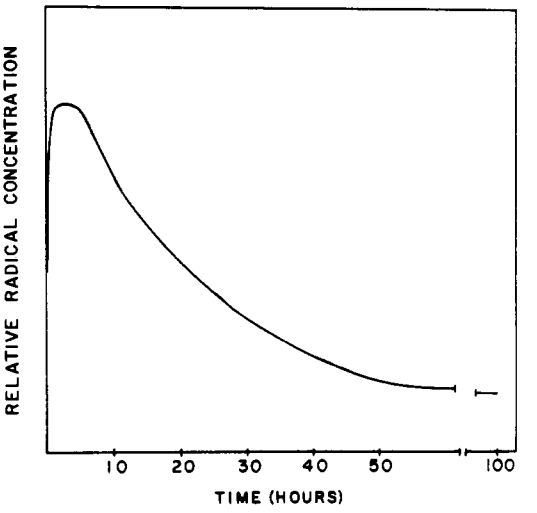

FIGURE 7: Relative concentration of radicals during the autoxidation of 6-hydroxybenzo[a]pyrene (0.52 mM) in 1:1 (v/v) 95% ethanol-0.01 M sodium phosphate (pH 7.0) at 22°.

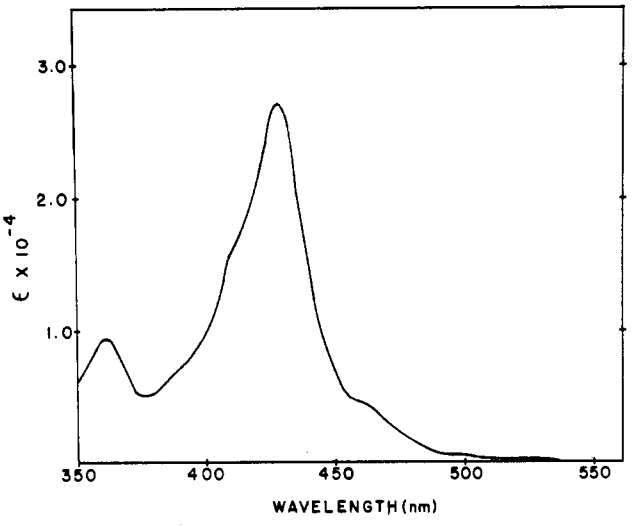

FIGURE 8: Absorption spectrum and the extinction coefficients of 6-oxobenzo[a]pyrene radical in benzene.

the electron interacts with four protons with approximately equal coupling constants. At lower modulations additional hyperfine couplings from other protons are observed. This ESR signal has been previously identified by Inomata and Nagata (1972) to be that of the 6-oxo-B[a]P free radical, a species obtained by the one-electron oxidation of 6-OH-B[a]P. Their molecular orbital analysis of the 6-oxo-B[a]P radical structure revealed that four positions in the molecule should have high and approximately equal spin densities leading to a basic five-line spectrum. The signal shows a line width phenomenon in aqueous ethanol but not in benzene solution. The outermost lines are broadened relative to the inner lines. The reason for this has not been determined, but the possibility of an intermolecular exchange process is suggested by the work on other systems (see Carrington and McLachlan, 1967).

Figure 7 presents the time course of the observed concentration of free radicals during the autoxidation of 6-OH-B[a]P in 1:1 buffer–ethanol solution. Since the 6-oxo-B[a]P radical is quenched by ordinary fluorescent lighting, all measurements have been made in the dark or in dim red light. The observed free radical signal at the later stages of the autoxidation is a mixture of the signal of the 6-oxo-B[a]P radical and the singlet signal of the unidentified radical described earlier, which persists after the decay of the 6-oxo-B[a]P radical. These two signals, however, can be distinguished readily from the line shape of the ESR signal because of the characteristic hyperfine structure of the 6-oxo-B[a]P radical. In the first 3–4 hr of the autoxidation, the ESR signal is mainly that of the 6-oxo-B[a]P radical. The change in line shape after this time is apparently due to the combination of the decrease of the 6-oxo-B[a]P radical signal and the gradual increase of the singlet signal of the unknown radical, which eventually becomes the only radical left in solution. The possibility of the presence of other free radicals which lack hyperfine structure at these oxygen concentrations cannot be excluded, however.

Properties of the 6-Oxo-B[a]P Radical. The 6-oxo-B[a]P radical was found to be relatively more stable dissolved in benzene than in other polar solvents. A procedure has been developed which quantitatively converts all the 6-OH-B[a]P to the 6-oxo-B[a]P radical in the absence of oxygen. 6-OH-B[a]P dissolved in benzene was shaken with an aqueous solution containing an excess quantity of a one-electron oxidizing agent, $K_3Fe(CN)_6$ (see Procedures). This procedure quantitatively, selectively, and rapidly converts all the starting 6-OH-B[a]P in benzene to the 6-oxo-B[a]P radical as determined by accurate ESR measurement. Figure 8 shows the absorption spectrum of the 6-oxo-B[a]P

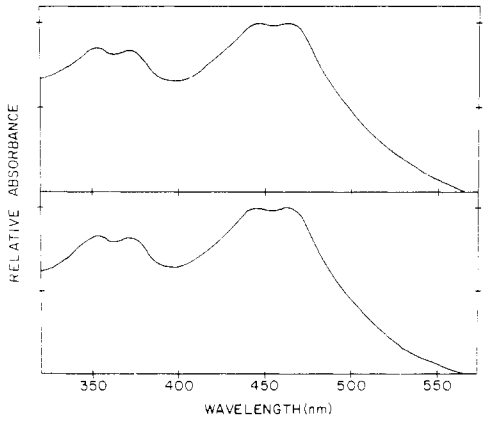

FIGURE 9: Comparison of the absorption spectra of the mixtures of the final products of the autoxidations of 6-hydroxybenzo[*a*]pyrene (top) and 6-oxobenzo[*a*]pyrene radical (bottom) in 1:1 (v/v) 95% ethanol–0.01 *M* sodium phosphate (pH 7.0).

radical, prepared in this manner in benzene. The benzene solution of the 6-oxo-B[*a*]P radical can be evaporated to dryness. Redissolution of the residue into benzene restores the ESR signal to its original intensity, provided that the original solution has been dried with a desiccant (Na_2SO_4) and nitrogen sparged. Not taking these precautions results in the loss of 5–10% of the intensity of the ESR signal. The solid 6-oxo-B[*a*]P radical, dissolved in 1:1 (v/v) 95% ethanol–0.01 *M* sodium phosphate (pH 7.0), was oxidized by air to the same three B[*a*]P diones and the same minor, unidentified material with a singlet ESR signal, as was obtained directly from the autoxidation of 6-OH-B[*a*]P itself. Figure 9 is a comparison of the absorption spectra of the final reaction solutions obtained in the autoxidations of 6-OH-B[*a*]P and 6-oxo-B[*a*]P radical under the same conditions. Their two spectra are virtually identical, indicating that the distribution of products is the same in both cases. This observation, together with other evidence, provides a strong support for the notion that 6-oxo-B[*a*]P radical is an obligatory intermediate in the autoxidation of 6-OH-B[*a*]P to the three B[*a*]P diones and the as yet unidentified, minor product.

Oxidation of 6-OH-B[a]P in Rat Liver Homogenates. 6-OH-B[*a*]P was incubated for 40 min in rat liver homogenate at 37°. After incubation, the homogenate was extracted with benzene, and the benzene extract was chromatographed and analyzed as described above. Only 60–70% of the material was recovered from the homogenate. Fifteen percent of the recovered material was 6,12-B[*a*]P dione; 41% was 1,6-B[*a*]P dione; and 44% was 3,6-B[*a*]P dione. The violet-colored, orange fluorescent material possessing a singlet ESR signal was not detected in the extracts by chromatographic analyses or ESR measurement.

The rate of oxidation of 6-OH-B[*a*]P in the liver homogenate can be followed most conveniently by ESR measurement of the 6-oxo-B[*a*]P radical in benzene extracts. In this procedure, the 6-OH-B[*a*]P in the liver homogenate was first extracted into benzene and then quantitatively converted to the 6-oxo-B[*a*]P radical for ESR measurement by shaking with aqueous $K_3Fe(CN)_6$. This procedure is described in detail in the accompanying paper (Lesko et al., 1975). The data indicate that the overall rate of 6-OH-B[*a*]P oxidation is considerably more rapid in homogenate than in 1:1 ethanol–phosphate buffer solution and has a half-time of about 2.5 min at 37°.

Discussion

While it is generally understood that autoxidations take place by one-electron transfer steps involving free radical intermediates (Taube, 1965), the mechanisms for the autoxidation of phenolic compounds are complex and not often understood in detail. When serving as an electron or hydrogen atom acceptor, molecular oxygen is univalently reduced according to this scheme:

$$O_2 \xrightarrow{+e} O_2\cdot^- \xrightarrow[+2H^+]{+e} H_2O_2 \xrightarrow[+H^+]{+e} {}^{\cdot}OH + H_2O \xrightarrow{+e,\ +H^+} H_2O$$

Due to dismutation and interconversion (Haber and Weiss, 1934), all of the three reactive intermediates, superoxide anion radical, hydrogen peroxide, and hydroxyl radical, are produced during the univalent reduction of oxygen. The spontaneous, nonenzymic dismutation of superoxide anion radical produces excited state singlet molecular oxygen as well (Mayeda and Bard, 1974; Schaap et al., 1974). The consumption of molecular oxygen has been demonstrated in the autoxidation of 6-OH-B[*a*]P in ethanol–aqueous buffer solutions. The formation of substantial amounts of hydrogen peroxide ($>2 \times 10^{-4}$ *M* max concentration, starting with 5×10^{-4} *M* 6-OH-B[*a*]P) verifies that oxygen is reduced by electron acceptance. It should be noted that the present measurement of hydrogen peroxide by an horseradish peroxidase assay is highly specific (Paul, 1963); potential organic peroxide intermediates discussed in later paragraphs are not likely to interfere with the assay.

Figure 10 presents a reaction scheme which is based on the one constructed by Jeftic and Adams (1970) from their study on the anodic oxidation of B[*a*]P to B[*a*]P diones. Previous work has tended to support the initial steps in this scheme showing a radical-cation intermediate and a preference for the 6 position during the initial chemical oxidation and nucleophilic substitution steps. The ESR spectrum of the radical-cation can be obtained by dissolving B[*a*]P in concentrated sulfuric acid (Nagata et al., 1974). Its presence is supported by the observations that oxidation of B[*a*]P by iodine in the presence of pyridine produces the 6-substituted pyridinium salt (Rochlitz, 1967) and oxidation by lead tetraacetate in the presence of acetic acid gives high yields of the 6-acetoxy substitution product (Fieser and Hershberg, 1938). In addition, mild oxidation of B[*a*]P by iodine in ethanol–aqueous buffer solution produces a reactive free radical which has been postulated to be the B[*a*]P cation-radical (Girke and Wilk, 1974; Caspary et al., 1973), even though the ESR signal does not exhibit a hyperfine structure needed for positive identification.

The data presented in this communication concern the subsequent four-electron oxidation from 6-OH-B[*a*]P to the three B[*a*]P diones (6,12, 1,6, and 3,6). In this scheme (Figure 10), the electrons are transfered directly to the oxidizing agent, in this case molecular oxygen or partially reduced forms. The first intermediate, the 6-oxo-B[*a*]P radical, observed and identified previously by Nagata et al. (1974) has been shown by our results to be an obligatory intermediate in the autoxidation of 6-OH-B[*a*]P to B[*a*]P diones. The second intermediate, the 6-oxobenzo[*a*]pyrenyl cation resulting from two such transfer steps, is hydrated by the solvent at its three most reactive positions as calculated by HMO (Jeftic and Adams, 1970). Oxonium ions of this type are known to exist and have been produced from oxo radicals; some are sufficiently stable to have been isolated as salts (Dimroth et al., 1967). The hydroquinones resulting

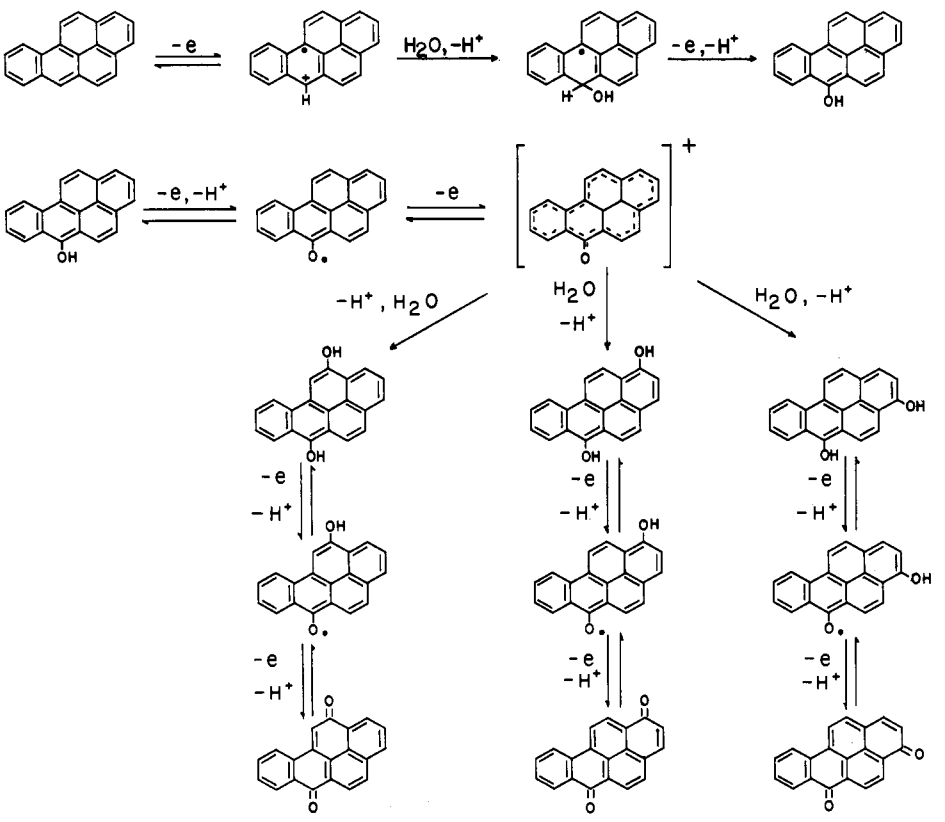

FIGURE 10: Reaction scheme proposed for the oxidation of benzo[a]pyrene to benzo[a]pyrene diones, after Jeftic and Adams (1970).

from hydration of this cation could then be oxidized to B[a]P diones by the univalent steps familiar in hydroquinone–quinone couples.

Another role for molecular oxygen in autoxidations is direct addition to free radical intermediates. Figure 11 presents an alternative reaction scheme for the production of B[a]P diones which has different intermediates subsequent to the production of the 6-oxo-B[a]P radical. In this alternate scheme, molecular oxygen couples directly with the 6-oxo-B[a]P radical at the positions of high spin density (Inomata and Nagata, 1972) producing ketoperoxyl radicals. Several subsequent pathways from the ketoperoxyl radicals to B[a]P diones are conceivable. The most likely, shown in Figure 11, would involve the formation of hydroperoxides or disubstituted peroxides, followed by the decomposition of these peroxides to produce B[a]P diones. In support of this scheme is the observation that many oxo radicals derived from substituted phenols produce ketoperoxides upon exposure to air (Forrester et al., 1968). Whether hydroperoxides or disubstituted peroxides are formed appears to depend upon the availability of hydrogen atom donors, which favors hydroperoxide formation, as well as the stability of the oxo radical, which favors disubstituted peroxide formation. In addition, decomposition of ketoperoxides under mild conditions has been shown to produce benzoquinones (Müller and Ley, 1955).

There are two important areas of contrast in these schemes. First, the source of the additional oxygen atom in the product B[a]P diones is the solvent (water) according to Figure 10, whereas it is molecular oxygen according to Figure 11. Appropriate experiments with oxygen isotopes will determine the source of the additional oxygen atom and provide more information about the reactive intermediates present during the oxidation of 6-OH-B[a]P. Secondly, given the different reactive intermediates suggested by each scheme, different products from the reaction of 6-OH-B[a]P with nucleic acid would be anticipated, a point which will be discussed in more detail, shortly.

Among the final products of the oxidation of 6-OH-B[a]P shown in the schemes, the 1,6- and 3,6-B[a]P diones have long been identified as products of B[a]P metabolism in both in vivo and in vitro experiments, whereas the 6,12-B[a]P was seldom reported (Falk et al., 1962). However, the recent application of high-pressure liquid chromatography, with its attendant high resolution and sensitivity, has resulted in the identification of the 6,12-B[a]P dione (Selkirk et al., 1974). Our studies demonstrate that 6-OH-B[a]P, incubated either in rat liver homogenate or in aqueous–ethanol solution, produces these same three B[a]P diones. The oxidation of 6-OH-B[a]P in liver homogenate was found not to be affected by a prior heating of the enzyme preparation or by witholding the addition of NADPH (Lesko et al., 1975). This observation suggests that the rapid oxidation of 6-OH-B[a]P in the homogenate may be chemical and not enzymic in nature. The isolated yield of 6,12-B[a]P dione from the homogenate incubation is the smallest of the three B[a]P diones. Sims (1967) reported that the 1,6- and 3,6-B[a]P diones were the only recognizable products of the incubation of 6-OH-B[a]P in rat liver homogenate. The infrequent detection of 6,12-B[a]P dione in B[a]P metabolic experiments may simply be a consequence of the low yield of this dione from 6-OH-B[a]P oxidation.

These B[a]P diones could, of course, arise from the oxidation, either chemical or enzymic, of other monooxygenated precursors, for instance, the 1- and 3- or the 12-hydroxy B[a]P. It has been reported that 3-OH-B[a]P produces the 3,6-B[a]P dione upon incubation with rat liver microsomes

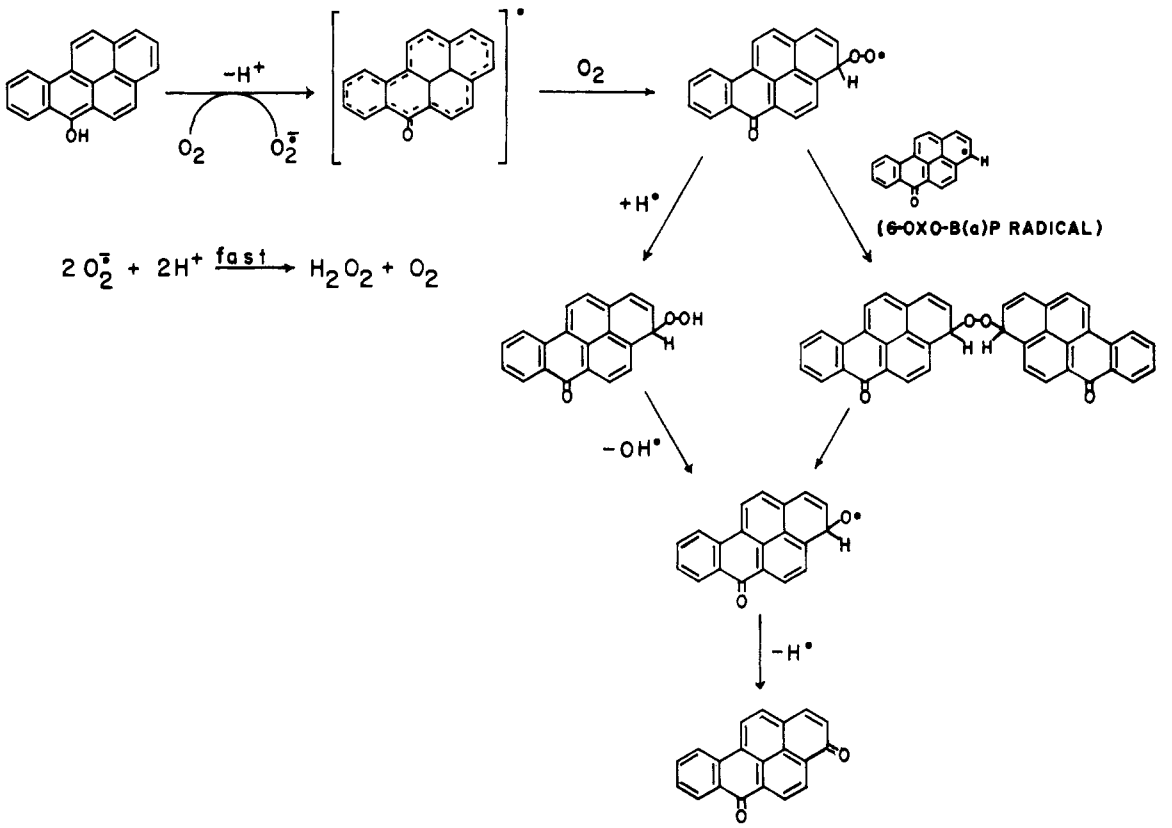

FIGURE 11: Alternative reaction scheme for the autoxidation of 6-OH-B[*a*]P to B[*a*]P diones. For the sake of brevity, the formation of only the 3,6-B[*a*]P dione is shown.

(Kinoshita et al., 1973). However, 3-OH-B[*a*]P is recognized as a major isolated B[*a*]P metabolite in the microsome system. In keeping with this, our studies show that 3-OH-B[*a*]P is more difficult to oxidize than 6-OH-B[*a*]P. In contrast to 6-OH-B[*a*]P, the solid 3-OH-B[*a*]P neither noticeably darkens upon exposure to air nor produces its phenoxy free radical when dissolved in air-saturated solution. While benzene solutions of the 3-oxo-B[*a*]P radical and the 6-oxo-B[*a*]P radical can be produced from their respective phenols by shaking with aqueous $K_3Fe(CN)_6$, only the 6-oxo-B[*a*]P radical can be found by shaking the phenols with the weaker oxidizing agent, 2,6-dichloroindophenol. These observations, together with the result reported in our accompanying paper (Lesko et al., 1975) that about 15–20% of the B[*a*]P metabolism in rat liver homogenate goes through the 6-OH-B[*a*]P pathway, clearly indicate that a substantial portion of the three B[*a*]P diones formed in the metabolism of B[*a*]P in rat liver homogenate must originate from the oxidation of 6-OH-B[*a*]P. The reported values of 18–21% conversion to B[*a*]P dione products found for the metabolism of B[*a*]P with rat liver microsomes (Kinoshita et al., 1973) agree well with the above observations and conclusions.

Preliminary experiments suggest that 6-OH-B[*a*]P, the 6-oxo-B[*a*]P radical, and the active reduced oxygen species (such as H_2O_2) can have serious biochemical and biological consequences (Ts'o et al., 1974). 6-OH-B[*a*]P reacts spontaneously and covalently with nucleic acids in ethanol-aqueous buffer solutions. The kinetics of covalent binding to poly(A) are comparable to the lifetime of the 6-oxo-B[*a*]P radical in solution. It may be that this radical is the species that reacts directly with the nucleic acid. The 6-oxo-B[*a*]P radical can be prepared and prevented from further oxidation to other reactive intermediates by removing oxygen. Thus, its reactivity, alone, toward nucleic acid can be determined. The further oxidized electrophilic intermediates proposed in the two oxidation schemes are also potentially reactive toward nucleic acid. For example, the 6-oxobenzo[*a*]pyrenyl cation, proposed in Figure 10, can be visualized as an "alkylating agent" and might be expected to react readily at positions 1, 3, and 12 with the nitrogen (most probable), oxygen, carbon, or even phosphate groups of nucleic acid. In contrast, the ketoperoxyl and the ketoalkoxyl radicals proposed in Figure 11 are likely to abstract hydrogen from nucleic acids, a process which probably initiates breakdown and cross-linking of nucleic acid rather than covalent attachment. Covalent binding could take place by the addition of these radicals, as well as the 6-oxo-B[*a*]P radical, to the double bonds in pyrimidine bases; there is also the possibility for small amounts of coupling between these radicals and radicals of nucleic acid formed during the reaction.

Incubation with 6-OH-B[*a*]P in ethanol-aqueous buffer solution also induces strand breakage of nucleic acid. These strand breaks are probably caused by hydrogen peroxide, organic peroxides, or other reduced oxygen species generated during autoxidation. Hydrogen peroxide or peroxide and hydroxyl radical generators are known to cause DNA strand breaks, base modifications, cross-linking, and inactivation of transforming principles (Freese et al., 1967; Melzer, 1967; Rhaese and Freese, 1968; Rhaese et al., 1968; Massie et al., 1972). Antioxidants have been used in various studies to reduce chromosomal breaks (Shamberger et al., 1973) and to decrease neoplasia incidences caused by chemical carcinogens (Wattenberg, 1973). However, it is not known whether the effect of the antioxidants in these exper-

iments is directly due to their elimination of reactive oxygen species or, indirectly, to their influence on the microsomal enzymes.

Finally, it should be noted that 6-OH-B[a]P is very toxic to human and hamster cells in culture and can induce in vitro morphological transformation of Syrian hamster embryonic fibroblasts (Schechtman et al., 1974). The biological role of the intermediates produced by the autoxidation of 6-OH-B[a]P will be tested for their activity in these cellular systems.

Acknowledgments

The 3-hydroxybenzo[a]pyrene was a generous gift of Dr. H. V. Gelboin. The authors thank Dr. P. C. Thorstenson for helpful discussions. Special thanks are given to Dr. R. G. Harvey for suggestions concerning the reaction mechanisms. Mass spectra were preformed by the laboratory of Dr. C. C. Fenslau.

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