Enzymic Formation of 6-Oxobenzo[*a*]pyrene Radical in Rat Liver Homogenates from Carcinogenic Benzo[*a*]pyrene†

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ABSTRACT: Upon incubation of benzo[*a*]pyrene (B[*a*]P) at 37° in rat liver homogenates fortified with NADPH generating cofactors, a metabolite was formed which gave rise spontaneously to an electron paramagnetic resonance (EPR) signal. The radical and its metabolic precursor were extracted into benzene for measurement, in which they are relatively stable. The EPR signal was identical with that extracted after incubating synthetic 6-hydroxybenzo[*a*]pyrene (6-OH-B[*a*]P) in rat liver homogenates and has been identified as the 6-oxobenzo[*a*]pyrene radical by its characteristic hyperfine structure. No EPR signal was detected when benzo[*a*]pyrene or cofactors were eliminated from the incubation mixture or when the homogenate was heated. The radical concentration reached a peak after 14–15 min of incubation and declined rapidly to a low level at 20 min. The decay of 6-hydroxybenzo[*a*]pyrene in rat liver homogenate did not appear to be enzymic and showed a first-order rate constant of 0.29 min^{-1} and a half-life of 2.4 min. The only significant products formed by oxidation of 6-hydroxybenzo[*a*]pyrene in rat liver homogenates were: 6,12-B[*a*]P dione (15%), 1,6-B[*a*]P dione (41%), and 3,6-B[*a*]P dione (44%). The percentage of B[*a*]P metabolized through the 6-hydroxybenzo[*a*]pyrene pathway can be estimated by comparing the initial rate of total benzo[*a*]pyrene metabolism in the aryl hydrocarbon hydroxylase system with the initial rate of 6-hydroxybenzo[*a*]pyrene formation. The amount of 6-OH-B[*a*]P produced was determined by measuring the 6-oxobenzo[*a*]pyrene radical concentration after a selective oxidation with 2,6-dichloroindophenol. The initial rate constants for 6-OH-B[*a*]P formation (per mg of protein) in liver homogenates of two species of rat were calculated after an appropriate correction for the lability of the compound. Formation of 6-OH-B[*a*]P was found to represent about 18 and 20% of total metabolism for female Sprague-Dawley rats and female ACI rats, respectively. The data indicate that 6-OH-B[*a*]P is a major metabolite of benzo[*a*]pyrene. This metabolite is very labile, being readily oxidized to the 6-oxobenzo[*a*]pyrene radical which is a transient intermediate in the further oxidation to quinones.

A considerable amount of evidence has accumulated in support of the hypothesis that carcinogenic polycyclic aromatic hydrocarbons require metabolic activation before they can neoplastically transform target cells. The environmental carcinogen, benzo[*a*]pyrene (B[*a*]P),[1] is metabolized to many identifiable, stable products by liver microsomes (Conney et al., 1957; Sims, 1967; Kinoshita et al., 1973; Holder et al., 1974; Selkirk et al., 1974) as well as by other in vitro (Rasmussen and Wang, 1974; Booth et al., 1974) and in vivo systems (Berenblum et al., 1943; Weigert and Mottran, 1946). The formation of the 6-oxo-B[*a*]P radical in rat liver homogenates has been reported by Nagata et al. (1968). However, 6-OH-B[*a*]P often has not been reported as a metabolite of B[*a*]P although its formation has been implied by the detection of the 6-OH-B[*a*]P glucuronide in an in vivo system (Falk et al., 1962). These investigators analyzed the products in the bile of rats in which [^{14}C]B[*a*]P had been injected intravenously, and found that the stable 6-OH-B[*a*]P glucuronide represented about 60% of the total metabolic yield in the first few hours. They concluded that both 3-OH-B[*a*]P and 6-OH-B[*a*]P were the most significant primary oxidation products.

We have synthesized 6-OH-B[*a*]P from B[*a*]P using modifications of the procedure of Fieser and Hershberg (1939). As described in the accompanying paper (Lorentzen et al., 1975), 6-OH-B[*a*]P is a very labile compound which spontaneously forms the 6-oxo-B[*a*]P radical in solution and covalently links to DNA (Ts'o et al., 1974). Here we report a substantial conversion of B[*a*]P to 6-OH-B[*a*]P by the enzyme systems in rat liver homogenates. The quantity of 6-OH-B[*a*]P was followed by the spontaneous formation of the 6-oxo-B[*a*]P radical as monitored by electron paramagnetic resonance (EPR) spectroscopy.

Experimental Section

Materials

B[*a*]P was purchased from Eastman Organic Chemicals, Rochester, N.Y., and Aldrich Chemical Co., Milwaukee, Wis., and was used without further purification. B[*e*]P was purchased from Adams Chemical Co., Round Lake, Ill., and General Biochemicals, Cleveland, Ohio. It was used as purchased after examination by thin-layer chromatography (TLC). Reagent grade benzene was purchased from Mallinckrodt, St. Louis, Mo., and further purified by distillation from charcoal and then passing it through an aluminum oxide–charcoal column. Generally labeled [^{3}H]B[*a*]P (25 Ci/mmol) and [^{3}H]B[*e*]P (4.5 Ci/mmol) were purchased from Amersham/Searle, Arlington Heights, Ill. Each hydrocarbon was diluted with nonradioactive material before use with ethanol–acetone (3:2) to the following specific activities: [^{3}H]B[*a*]P (3.3 Ci/mol) and [^{3}H]B[*e*]P (0.37 Ci/mol). The 6-OH-B[*a*]P was prepared by a modifi-

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

cation of the procedure of Fieser and Hershberg (1939). After reductive cleavage of 6-acetoxy-B[a]P, the 6-OH-B[a]P was purified by vacuum sublimation at 160–170° (Lorentzen et al., 1975). Dr. H. V. Gelboin of NIH generously supplied the purified 3-OH-B[a]P.

Methods

Preparation of Homogenates. Livers were obtained from various strains of adult rats immediately after asphyxiation in ether. The livers were washed with cold 1.15% KCl and stored at −80°. Homogenates were prepared by a method similar to that of Nagata et al. (1968). Livers were placed in 1.15% KCl at 0° to thaw, then cut into small pieces and homogenized at 0° in 1.15% KCl with a motor-driven Potter-Elvehjem glass–Teflon homogenizer (5–6 g of liver in 15 ml). Homogenates were centrifuged at 1500*g* for 20 min at 5° and the supernatant was used as a source of enzyme.

Assay for Metabolic Formation of 6-Oxo-B[a]P Radical. A procedure similar to that of Nagata et al. (1968) was followed. For each experimental point 15 ml of homogenate (30–35 mg of protein/ml) was added to 15 ml of 0.1 *M* potassium phosphate buffer (pH 7.4) containing cofactors for generating NADPH. The reaction mixture contained 88 m*M* niacinamide, 0.22 m*M* NADP, and 5.4 m*M* glucose 6-phosphate. After a preincubation at 37° for 2 min, hydrocarbon was added in 0.25 ml of acetone. The reaction mixture was then incubated at 37° aerobically by mechanical shaking in a 250-ml erlenmeyer flask. The reaction was stopped by adding the incubation mixture to 0.5 ml of 10% sodium dodecyl sulfate in a Waring Blendor and extracting the hydrocarbon by homogenizing with 200 ml of cold benzene for 2 min at 30 V. This mixture was placed in a separatory funnel and allowed to stand 15 min for phase separation. The benzene phase was filtered through Whatman No. 1 paper and taken to dryness under reduced pressure after which the residue was dissolved in 1.5 ml of benzene for EPR measurement. No EPR signal could be found from a second benzene extraction of the reaction mixture in the Waring Blendor. The samples were examined at room temperature in a Jeol JES-ME-1X EPR spectrometer. Concentrations were obtained by comparing double integrations of signals from a MnO standard and the 6-oxo-B[a]P radical.

Assay for Aryl Hydrocarbon Hydroxylase in Rat Liver Homogenate. A procedure similar to that of Hayakawa and Udenfriend (1973) was followed. For each experimental point 0.5 ml of homogenate (32–40 mg of protein/ml) was added to 0.5 ml of 0.1 *M* potassium phosphate buffer (pH 7.4) containing NADPH generating cofactors. Conditions were identical with those described in the above section. After a preincubation at 37° for 1 min, 12.5 μl of tritiated hydrocarbon was added. The reaction mixture was incubated at 37° aerobically by mechanical shaking. The reaction was stopped by adding 50 μl of 5 *N* $HClO_4$ and placing the sample on ice. After centrifugation, the supernatant was passed through a disposable activated charcoal column. The pellet was rinsed with 0.4 ml of 0.25 *N* $HClO_4$ twice and the washings were applied to the column. All the effluent from the column was collected in a counting vial and mixed with about 20 ml of scintillation fluid (Hydromix, Yorktown Research, New Hyde Park, N.Y.). The amount of B[a]P hydroxylated was calculated by measuring cpm in the tritiated water and correcting this value with an appropriate quench factor. The calculation was based on the assumption that all the tritium atoms were distributed equally on the B[a]P ring system.

Oxidation of 6-OH-B[a]P to 6-Oxo-B[a]P Radical. Quantitative oxidation of 6-OH-B[a]P to 6-oxo-B[a]P radical was accomplished by mechanically shaking 6-OH-B[a]P in 1.5 ml of benzene with 1 ml of 10 m*M* $K_3Fe(CN)_6$ in aqueous solution for approximately 5 min (Lorentzen et al., 1975). However, this procedure also oxidizes other B[a]P metabolites to various radicals. For instance, 3-OH-B[a]P can be oxidized by $K_3Fe(CN)_6$ to produce a radical signal that differs from the 6-oxo-B[a]P radical signal (unpublished data). On the other hand, selective and quantitative oxidation of 6-OH-B[a]P to the 6-oxo-B[a]P radical was accomplished by mechanically shaking a 1.5-ml benzene extract containing B[a]P and various metabolites with 1 ml of 10 m*M* 2,6-dichloroindophenol in aqueous solution. Samples were centrifuged for phase separation before EPR measurement of benzene layer. Under this latter condition only the characteristic hyperfine structure of the 6-oxo-B[a]P radical was seen (Nagata et al., 1968) and 3-OH-B[a]P did not give rise to an EPR signal.

Other Procedures. Protein was determined by a modified Lowry procedure (Hartree, 1972) using bovine serum albumin as a standard. The oxidative products of 6-OH-B[a]P extracted into benzene from rat liver homogenates were separated by aluminum oxide chromatography (Lorentzen et al., 1975). In order to obtain the hyperfine structure of the EPR signals in benzene, the solutions were extensively sparged with N_2.

Results

Free Radical Produced by Incubating B[a]P with Rat Liver Homogenate. Upon incubation of B[a]P in uninduced rat liver homogenates fortified with a NADPH-generating system at 37° a metabolite was formed which gave rise spontaneously to an EPR signal. The metabolite and the radical can be extracted quantitatively into benzene in which they are relatively stable. The metabolite also can be quantitatively converted to the radical by shaking with aqueous solutions of 2,6-dichloroindophenol or $K_3Fe(CN)_6$. The latter compound, however, is not selective in its action and will also oxidize the 3-OH-B[a]P to its oxo radical. The EPR measurements of the quantities of radical were done in benzene after the extracted solution had been taken to dryness under vacuum and redissolved in a small volume. The EPR signal was identical with that extracted after incubating 6-OH-B[a]P in fortified rat liver homogenates (Figure 1) and has been identified as the 6-oxo-B[a]P radical by its characteristic hyperfine structure (Nagata et al., 1968; Lorentzen et al., 1975). Identical hyperfine spectra were obtained before and after oxidation with 2,6-dichloroindophenol. No EPR signal was seen when B[a]P or cofactors were eliminated from the incubation mixture or when the homogenate was heated at 65° for 10 min. These observations indicate that the oxidation process of B[a]P to 6-OH-B[a]P is enzymic in nature. Figure 2 shows the effect of protein concentration on radical production. Increasing amounts of homogenate resulted in higher concentrations of 6-oxo-B[a]P radical being produced.

Kinetics of 6-oxo-B[a]P radical formation in liver homogenates show an initial increase peaking at 14–15 min followed by a rapid decline to a low concentration at 20 min which persisted up to at least 40 min (Figure 3). The homogenates from Sprague-Dawley and ACI rats showed similar kinetics in which the radical concentration peaked around 14–15 min. However, with CFN rats, the radical concentration did not increase after 2 min of incubation al-

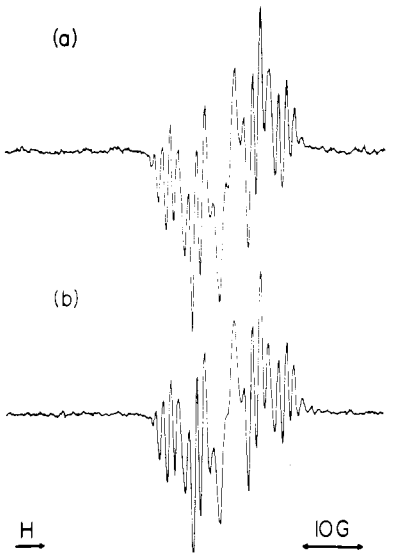

FIGURE 1: EPR spectra of 6-oxo-B[*a*]P radicals in benzene solutions measured at room temperature after extensive sparging with nitrogen. The radicals were obtained by incubating B[*a*]P or 6-OH-B[*a*]P at 37° in fortified rat liver homogenates, after which they were extracted into benzene. The extracted products in benzene were oxidized by shaking with aqueous solutions of 2,6-dichloroindophenol. (a) B[*a*]P; (b) 6-OH-B[*a*]P. Power, 10 mW; mod. width, 0.5 G.

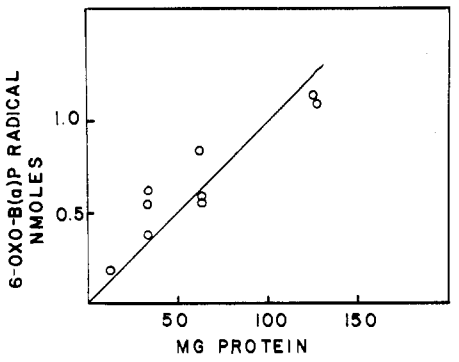

FIGURE 2: Dependence of 6-oxo-B[*a*]P radical formation from B[*a*]P on the amount of rat liver homogenate present in incubation mixture; 3, 1.5, 0.8, or 0.3 ml of female Buffalo rat liver homogenate, 15 ml of cofactors, 0.14 m*M* B[*a*]P (added after 2-min preincubation), and 1.15% KCl were incubated in 30 ml volume for 12 min at 37°. Samples were treated as described in Methods. Radical concentration was measured after oxidation of metabolite in benzene by shaking with aqueous 2,6-dichloroindophenol.

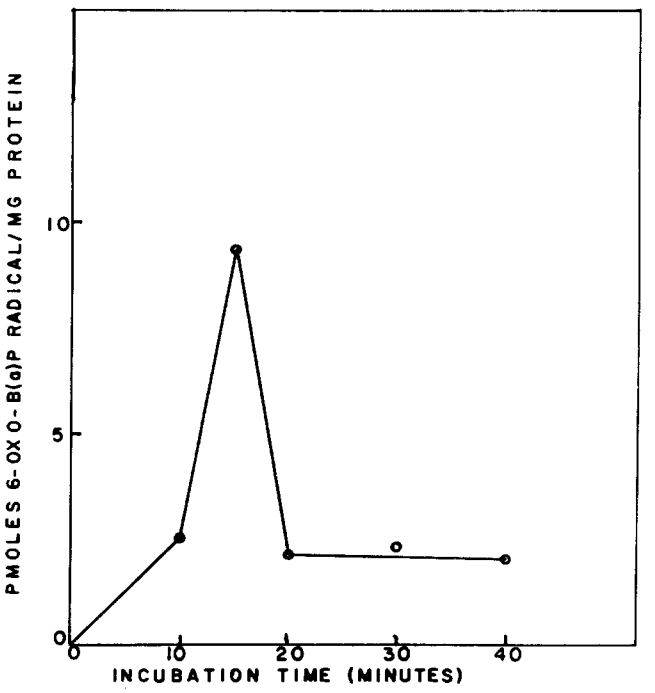

FIGURE 3: Kinetics of 6-oxo-B[*a*]P radical production from the incubation of B[*a*]P at 37° in fortified female ACI rat liver homogenates. Reaction mixtures contained 107 ml of homogenate, 107 ml of cofactors, and 0.68 m*M* B[*a*]P which was added after a 4-min preincubation at 37°. Incubation was done in a 1-l. erlenmeyer flask and 30-ml samples were removed, extracted, treated, and measured in the EPR spectrometer as described in Methods.

though the concentration at this time point was similar to that seen in the other strains.

B[*e*]P, a structural and noncarcinogenic analog of B[*a*]P, did not give an EPR signal when incubated 10, 20, or 40 min in a rat liver homogenate under conditions in which B[*a*]P was converted to a radical. No EPR signal was seen even after shaking the benzene extract with aqueous $K_3Fe(CN)_6$.

Oxidation of 6-OH-B[a]P in Rat Liver Homogenates. Figure 4 shows the kinetics of 6-OH-B[*a*]P in rat liver homogenates. After benzene extraction and treatment described in Methods, any unreacted 6-OH-B[*a*]P was quantitatively converted to 6-oxo-B[*a*]P radical with $K_3Fe(CN)_6$ and total radical concentration was measured by EPR. The decay of 6-OH-B[*a*]P in rat liver homogenates showed first-order kinetics with a rate constant of 0.29 min^{-1}. The half-life of 6-OH-B[*a*]P in this system was about 2.4 min. The rate of degradation of 6-OH-B[*a*]P was not decreased by either elimination of the NADPH-generating system or by heating the homogenate at 65° for 10 min, thus indicating that this reaction was nonenzymic in nature.

The products formed by incubating 6-OH-B[*a*]P in rat liver homogenates were extracted into benzene and isolated by aluminum oxide chromatography (Lorentzen et al., 1975). The data show that 6-OH-B[*a*]P was oxidized in this system and the following were the only products detected after 40 min of incubation: 6,12-B[*a*]P dione (15%), 1,6-B[*a*]P dione (41%), and 3,6-B[*a*]P dione (44%).

Determination of Rate Constant for B[a]P Hydroxylation in Rat Liver Homogenate. Reactions were followed by measuring release of tritium from the site of hydroxylation on the substrate as described by Hayakawa and Udenfriend (1973). Figure 5 shows the kinetics of hydroxylation of B[*a*]P at 37° in liver homogenates from uninduced adult female Sprague-Dawley and ACI rats. These reactions proceed essentially at constant velocity over the first 10 min with the characteristics of zero-order kinetics. The rate constants per milligram of protein calculated from the data presented in Figure 5 are 1.52×10^{-8} and 0.72×10^{-8} mol $l.^{-1}$ min^{-1} for Sprague-Dawley and ACI rats, respectively.

Hydroxylation of B[*e*]P also followed zero-order kinetics at 37° over the first 10 min of reaction and the calculated rate constant per milligram of protein is 0.9×10^{-8} mol $l.^{-1}$ min^{-1} for Sprague-Dawley female rats. It should be noted that while the rate of hydroxylation of B[*e*]P is only 40% lower than that of B[*a*]P, this noncarcinogene analog produces no detectable radical or radical-forming metabolite in contrast to the carcinogenic B[*a*]P.

Calculation of the Rate Constants for 6-OH-B[a]P For-

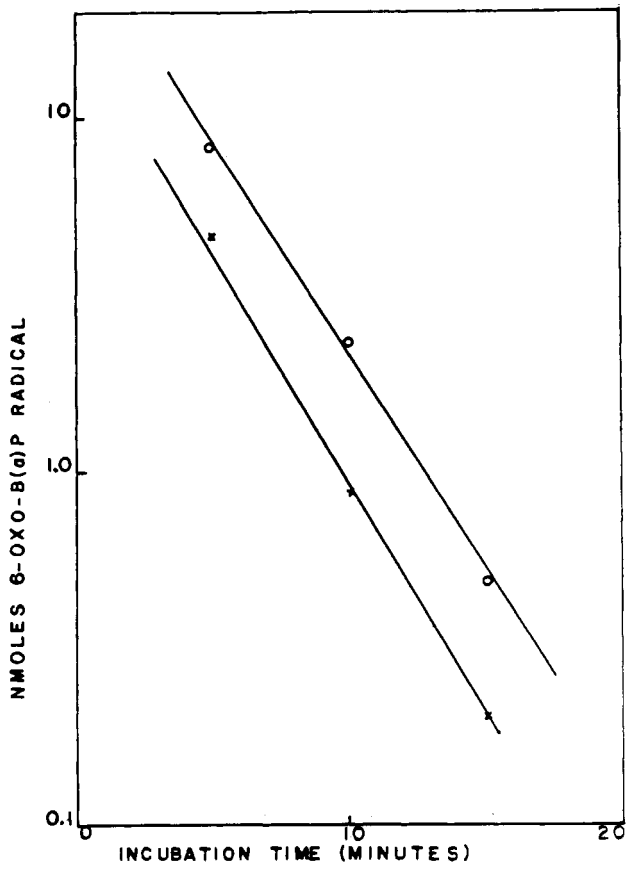

FIGURE 4: Kinetics of 6-OH-B[*a*]P degradation in fortified Sprague-Dawley male liver homogenates at 37°. Reactions contained 30 ml of homogenate, 30 ml of cofactors, and 6-OH-B[*a*]P added at 0°. Incubation was done in a 500-ml erlenmeyer flask and 15-ml samples were removed, treated, and measured after $K_3Fe(CN)_6$ oxidation, as described in Methods. Initial 6-OH-B[*a*]P concentration, 5.3 μM (x); 6.1 μM (O).

mation. The metabolic pathway of B[*a*]P to 6-OH-B[*a*]P and beyond can be schematically represented as follows:

$$\text{B[}a\text{]P} \xrightarrow{k_1} \text{6-OH-B[}a\text{]P} \xrightarrow{k_2} \text{6-oxo-B[}a\text{]P radical} \xrightarrow{k_3} \text{products}$$

In theory, the quantity of 6-OH-B[*a*]P and of the 6-oxo-B[*a*]P radical in the liver homogenates can be evaluated separately. A concentration of 6-oxo-B[*a*]P radical is obtained by first extracting both materials into benzene for the measurement. The benzene extract is then oxidized by shaking with aqueous 2,6-dichloroindophenol, which quantitatively converts all the 6-OH-B[*a*]P to 6-oxo-B[*a*]P radical. Thus, an EPR measurement of this oxidized extract provides the sum of the concentration of the original radical plus the radical derived from the oxidation of the 6-OH-B[*a*]P. The difference in the two EPR measurements before and after the oxidation by 2,6-dichloroindophenol should furnish the amount of 6-OH-B[*a*]P in the benzene extract. However, our results indicate that the procedures used to extract the radical from the homogenate also allow some air oxidation of the 6-OH-B[*a*]P to the radical. Consequently, the observed concentrations of the 6-oxo-B[*a*]P radical are not reliable estimates of the amount of radical in the homogenate and are variable from experiment to experiment. In contrast, the quantity of the 6-oxo-B[*a*]P radical in the benzene extract *after* the 2,6-dichloroindophenol oxidation is consistent and reliable. The ease of oxidation of 6-OH-B[*a*]P to 6-oxo-B[*a*]P radical suggests that the rate constant of this conversion (k_2) in the homogenate may be substantially greater than k_3, the rate constant describing the disappearance of radical. This notion is supported by the kinetics of oxidation of synthetic 6-OH-B[*a*]P in rat liver homogenate. The rate of formation of the 6-oxo-B[*a*]P radical is too rapid to be observed in 1 min whereas its decay can be monitored over a 15-min period. If k_2 is substantially greater than k_3 (fivefold or more), as is observed in the autoxidation of 6-OH-B[*a*]P in ethanol–buffer solution, then k_2 can be ignored and the reaction approximated by the following scheme:

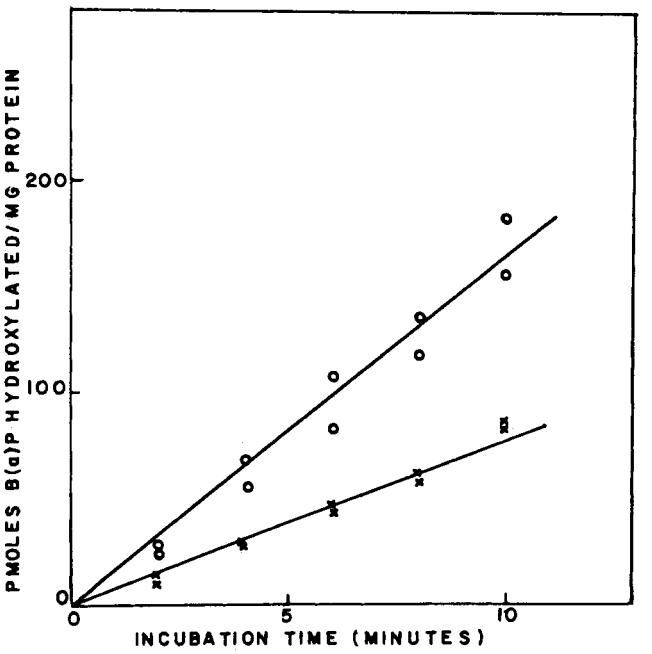

FIGURE 5: Kinetics of [^{3}H]B[*a*]P hydroxylation in fortified rat liver homogenates at 37°. Reaction mixtures contained 4 ml of homogenate, 4 ml of cofactors, and 0.11 mM [^{3}H]B[*a*]P added after 1-min preincubation; 1-ml samples were removed and added to 50 μl of cold 5 N $HClO_4$. The supernatent was treated and measured as described in Methods. Female Sprague-Dawley rats (O); female ACI rats (x).

$$\text{B[}a\text{]P} \xrightarrow{k_1} \text{R} \xrightarrow{k_3} \text{products}$$

where [R] = [6-OH-B[*a*]P] + [6-oxo-B[*a*]P radical], that is, the radical concentration after oxidation by 2,6-dichloroindophenol. The concentration of the radical [R], is subjected to the influence of two kinetic factors: (1) the zero-order rate constant, k_1, governing the formation of 6-OH-B[*a*]P under a saturating condition of B[*a*]P; (2) the first-order rate constant, k_3, governing the further oxidation of 6-oxo-B[*a*]P radical to the products. The concentration of [R] at any given time can be quantitatively described by the following equation:

$$d[R]/dt = k_1 - k_3[R] \quad (1)$$

which is a homogeneous linear differential equation. This differential equation can be solved to yield the value of the zero-order rate constant k_1 at time t, when [R] and k_3 can be independently measured as shown in eq 2:

$$k_1 = [R]k_3/(1 - e^{-k_3 t}) \quad (2)$$

The k_3 value of 0.29 min^{-1} was reported in a previous section.

Table I lists the rate constants, k_1, for 6-OH-B[*a*]P formation in the early period of incubation as calculated from eq 2 and the measurements of [R].

Table I: Zero-Order Rate Constants for 6-OH-B[*a*]P Formation at 37° in Uninduced Rat Liver Homogenates.[a,b]

	k_1 (mol l.$^{-1}$ min^{-1}) × 10^9/mg of Protein	
Time (min)	Sprague-Dawley Rat	ACI Rat
1.0	2.4, 4.2	2.8
1.5		1.1
3.0	1.8, 2.2	1.1
3.5		1.2
5.0	3.5, 2.5	1.4
7.0	4.4, 1.9	1.4

[a] Calculated from eq 2. [b] Reaction mixtures contained 62 ml of homogenate, 62 ml of cofactors, and 0.18 m*M* B[*a*]P which was added after a 2-min preincubation. Incubation done in a 500-ml erlenmeyer and 30-ml samples were removed for measurement of 6-oxo-B[*a*]P radical concentration after quantitative oxidation of benzene-extracted 6-OH-B[*a*]P by shaking with aqueous 2,6-dichloroindophenol solution.

The average values for the rate constants per milligram of protein are 2.8×10^{-9} and 1.5×10^{-9} mol l.$^{-1}$ min^{-1} for Sprague-Dawley and ACI rats, respectively. As shown in Figure 3, these values decrease rapidly after about a 12–15-min period of incubation.

Formation of Radicals by Other Carcinogenic Polycyclic Hydrocarbons in Rat Liver Homogenate. 3-Methylcholanthrene, 7,12-dimethylbenz[*a*] anthracene, or dibenz[*a,h*]-anthracene was incubated in uninduced rat liver homogenates, under conditions in which a radical was produced from B[*a*]P. The benzene extracts of the incubation mixtures were examined after 10 and 30 min of incubation for the presence of a radical. No EPR signals were detected in the incubation mixture with these three hydrocarbons even after mild oxidation of the benzene extractable products with aqueous solutions of $K_3Fe(CN)_6$. However, incubation of the strongly carcinogenic dibenzo[*a,h*]pyrene did give rise spontaneously to an EPR signal after incubation for 10 or 30 min. The EPR signal in benzene was a singlet and no hyperfine structure could be detected even after extensive sparging with N_2. The total metabolism of these four hydrocarbons have not been investigated under these conditions, however.

Discussion

The data presented here confirm the results of Nagata et al. (1968) that the 6-oxo-B[*a*]P radical is produced by incubating B[*a*]P in rat liver homogenates fortified with cofactors to generate NADPH. The kinetics we observe for radical production are very similar to those reported by Nagata et al. (1974) and indicate that the 6-oxo-B[*a*]P radical is a transient intermediate in the enzymic oxidation of B[*a*]P by rat liver homogenates. Our accompanying paper (Lorentzen et al., 1975) has shown that the 6-oxo-B[*a*]P radical is formed spontaneously from 6-OH-B[*a*]P and is an obligatory intermediate of the autoxidation of 6-OH-B[*a*]P to the B[*a*]P diones. However, the half-life of added 6-OH-B[*a*]P in rat liver homogenates is only 2.5 min and the kinetics of the formation of 6-OH-B[*a*]P from added B[*a*]P in rat liver homogenates exhibits an unusual time course of an early rise and rapid decline to a low concentration level (Figure 3). In addition, a recent study on the separation of B[*a*]P metabolites by high pressure liquid chromatography revealed that authentic samples of 2-OH-B[*a*]P, 6-OH-B[*a*]P, 8-OH-B[*a*]P, and 9-OH-B[*a*]P are not resolvable by this procedure (Holder et al., 1974). The above reasons may be responsible for 6-OH-B[*a*]P often not being reported as one of the B[*a*]P metabolites in homogenates or microsomal systems. The possible existence of 6-OH-B[*a*]P was mentioned in the discussions of several metabolic studies, however (Sims et al., 1967; Borgen et al., 1973; Kinoshita et al., 1973; Selkirk et al., 1974b).

We have identified the following three B[*a*]P diones as the only significant products formed by oxidation of 6-OH-B[*a*]P in rat liver homogenates: 6,12-B[*a*]P dione (15%), 1,6-B[*a*]P dione (41%), and 3,6-B[*a*]P dione (44%). These B[*a*]P diones have been reported as metabolites of B[*a*]P upon incubation with rat liver microsomes (Selkirk et al., 1974; Kinoshita et al., 1973; Holder et al., 1974) and our data would indicate that a significant portion of the B[*a*]P diones reported by these investigators results from oxidation of B[*a*]P through the 6-OH-B[*a*]P pathway. Additional investigation on the oxidation of 6-OH-B[*a*]P to the B[*a*]P diones would be of importance, although the rate of oxidation of 6-OH-B[*a*]P to the 6-oxo-B[*a*]P radical in rat liver homogenates is very fast, and was not reduced by heating the homogenate or by elimination of NADPH-generating cofactors. Currently, little is known about the oxidation process of 6-oxo-B[*a*]P radical to quinones in liver homogenates.

In the rat liver homogenates we are able to estimate the percentage of B[*a*]P metabolism proceeding through the 6-OH-B[*a*]P pathway at 37° by comparing the initial rate of total B[*a*]P metabolism with the initial rate of 6-OH-B[*a*]P formation. The determination of the latter rate is difficult because of the lability of 6-OH-B[*a*]P. Fortunately, 6-OH-B[*a*]P can be followed conveniently via the 6-oxo-B[*a*]P radical and corrections can thus be made for its lability. The total metabolism of [^{3}H]B[*a*]P was measured by the release of tritium catalyzed by the aryl hydrocarbon hydroxylase system (Hayakawa and Udenfriend, 1973). This study reported and discussed possible reasons for the radioassay giving higher values for the metabolism of B[*a*]P than the fluorometric assay of alkaline extractable metabolites described by Nebert and Gelboin (1968). Thus, the radioassay is less likely to lead to an underestimation of the total metabolism; data on the possible retention of tritium by NIH shifts during B[*a*]P metabolism are not available.

The percentage of total metabolism proceeding through 6-OH-B[*a*]P is about 18% for female Sprague-Dawley rats and about 20% for female ACI rats. The reaction rates were measured at relatively high substrate concentration to ensure zero-order kinetics. The values shown in Table I for 6-OH-B[*a*]P formation are constant within experimental error.

Another major primary metabolite of B[*a*]P in rat liver microsomes is 3-OH-B[*a*]P (Kinoshita et al., 1973). This compound has been used as the basis for a fluorometric assay of B[*a*]P hydroxylase (Nebert and Gelboin, 1968). Our experiments show that 3-OH-B[*a*]P is quite stable chemically and does not give rise to an EPR signal spontaneously when dissolved in benzene or even when oxidized by shaking with aqueous 2,6-dichloroindophenol. However, an EPR signal can be detected in benzene when 3-OH-B[*a*]P is oxidized by shaking with aqueous $K_3Fe(CN)_6$. The hyperfine spectrum observed from this radical is quite distinct from that observed from the 6-oxo-B[*a*]P radical. Nagata et al. (1974) have also obtained the hyperfine EPR spectrum of 3-OH-B[*a*]P by oxidation in dimethyl sulfoxide with ceric sulfate under alkaline conditions. There are reports that 3-OH-B[*a*]P is metabolized to the 3,6-B[*a*]P

dione in the rat (Falk et al., 1962) and in rat liver microsomes (Kinoshita et al., 1973), although there are no data to indicate that this oxidation proceeds through an oxo-radical intermediate.

B[e]P, a noncarcinogenic isomer of B[a]P, did not give rise to an EPR signal when incubated in rat liver homogenates. Under this condition of incubation, [^{3}H]B[e]P was found to be metabolized at a rate of about 60% that of [^{3}H]B[a]P. The benzene-extractable products of the incubation did not produce any radical even when oxidized by shaking with aqueous solutions of $K_3Fe(CN)_6$. Thus, the noncarcinogenic B[e]P does not appear to be metabolized via a pathway involving radical formation.

Preliminary investigation has been made on the metabolism of four other carcinogenic hydrocarbons under the same incubation condition in the rat liver homogenates in which B[a]P forms the 6-oxo-B[a]P radical. Radicals were not found in the benzene extracts of the incubation mixtures with 3-methylcholanthrene, 7,12-dimethylbenz[a]anthracene, and dibenz[a,h]anthracene even after oxidation by shaking with aqueous $K_3Fe(CN)_6$ solutions. Radical was found, however, in the liver homogenate incubation mixture with dibenzo[a,h]pyrene; this radical, for unknown reasons, does not show any hyperfine structure (a singlet) and, therefore, cannot yet be identified. The quantitative aspects of the metabolism of these four hydrocarbons in our rat liver homogenates will soon be investigated. Nagata et al. (1967) have detected EPR signals after stirring B[a]P and 3-methylcholanthrene with mouse skin homogenates at 4° for 5 days; in this system, 7,12-dimethylbenz[a]anthracene did not give an EPR signal.

Previous studies from this and other laboratories on the chemical activation of polycyclic hydrocarbon to become reactive species have implicated the participation of free radicals as intermediates. We reported (Hoffmann et al., 1970) an extensive I_2-induced covalent linkage of the carcinogens B[a]P, 3-methylcholanthrene, and 7,12-dimethylbenz[a]anthracene to DNA and polynucleotides in aqueous–ethanol solution. Under the same conditions, the noncarcinogens, B[e]P and benz[a]anthracene, reacted to a much lesser extent with DNA. Following this line of investigation, we (Caspary et al., 1973) were able to correlate the steady-state radical concentration induced by I_2 in benzene with the carcinogenicity of 14 polycyclic hydrocarbons. With only two exceptions, carcinogenic compounds gave significantly higher steady-state radical concentrations than noncarcinogens. Furthermore, additions of nucleosides quenched these radical signals. Pascal et al. (1971) have also reported the linkage of carcinogenic polycyclic hydrocarbon free radicals, produced indirectly by irradiation of I_2, to DNA. Wilk et al. (1966), Rochlitz (1967), as well as Girke and Wilk (1974) have continually advocated the important role of cation radicals in the chemical activation of B[a]P, especially in a mild oxidation reaction such as that induced by I_2.

According to the oxidation scheme proposed in the electrochemical study of Jeftic and Adams, and discussed in the preceding paper, the main radical species of B[a]P implicated in the I_2 studies mentioned above presumably would be the cation radical, the first intermediate in their anodic oxidation prior to the formation of 6-OH-B[a]P. Unfortunately, this assumption has not been verified because of the lack of hyperfine structure of the radical signal observed in the I_2 induced reaction. Furthermore, such a radical will be much more difficult to isolate from either a chemical or biological system. Therefore, the experimental emphasis of our laboratory has been shifted to the oxidation process starting with 6-OH-B[a]P as reported in these two communications. It is entirely possible that in the process of metabolic oxidation of B[a]P to 6-OH-B[a]P, there exists a B[a]P cation radical intermediate, as depicted in the electrochemical oxidation scheme of Jeftic and Adams (1970). The biological role of this cation radical if it exists is uncertain at present, although the evidence suggests that such a radical can be chemically rather reactive.

These investigations clearly indicate, however, that metabolic pathway leading to 6-OH-B[a]P is one of the major pathways of B[a]P metabolism in uninduced rat liver homogenates. In addition, 6-OH-B[a]P is spontaneously oxidized further to produce the 6-oxo-B[a]P radical. This radical can be identified and isolated and is an obligatory intermediate in the oxidation pathway to the three B[a]P diones (1,6 dione, 3,6 dione, and 6,12 dione) found in many biological systems as metabolites of B[a]P. It is interesting to note that a radical was not found in the present system for the metabolism of the noncarcinogenic B[e]P analog. In light of the observation that carcinogenic dibenzo[a,h]pyrene was metabolized to yield a free radical in the present study, it will be of interest to examine this system further.

We have reported previously that, in aqueous buffer–ethanol solution, 6-OH-B[a]P reacts covalently and spontaneously with DNA and polynucleotides and induces strand breaks in this process (Ts'o et al., 1974). Furthermore, 6-OH-B[a]P is very toxic to human and hamster cells in culture and preliminary studies show that it also causes morphological transformation of cultured Syrian hamster embryo fibroblasts (Schechtman et al., 1974). In this regard, Nagata et al. (1974) reported the production of fibrosarcomas in five of ten Donryu rats after subcutaneous injection of 6-OH-B[a]P in tricaprylin.

A particularly interesting observation of Nagata et al. (1974) was the detection of enzymic activity in the nucleus capable of converting considerable amounts of B[a]P to the 6-oxo-B[a]P radical, thereby enabling an active metabolite to be formed in the area of the potential DNA target molecule.

It should be noted that a definitive establishment of 6-OH-B[a]P and 6-oxo-B[a]P radical as proximate or ultimate carcinogens of B[a]P does not preclude the establishment of other B[a]P metabolites as proximate or ultimate carcinogens of B[a]P, and vice versa. Currently, there is little theoretical or experimental reason to believe a carcinogen such as B[a]P can be metabolized to yield only *one* form of proximate or ultimate carcinogen.

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Proton Nuclear Magnetic Resonance Studies of the Helix–Coil Transition of d-ApTpGpCpApT in D_2O Solution†

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ABSTRACT: The helix–coil transition of the self-complementary duplex d-ApTpGpCpApT between 5 and 70° has been monitored by following the nonexchangeable purine and pyrimidine resonances and the sugar H_1', resonances in D_2O, low ionic strength, pH 7. The resonances have been assigned on the basis of partial deuteration studies, from comparison of $T_{1/2}$ values and the temperature dependence of the chemical shift between 0 and 20°. The $T_{1/2}$ values increase in a sequential manner on proceeding from terminal to internal to central base pairs for the d-ApTpGpCpApT duplex. For a given base pair, the $T_{1/2}$ values are dependent on resonance position, and increase in the order pyrimidine 5 < sugar H_1' < purine 2. The differential melting curve of the central cytosine H_5 resonance yields a reaction enthalpy for the helix–coil transition of 34 kcal. The line width changes of the cytosine H_5 resonance at the $T_{1/2}$ of the helix–coil transition yields the kinetic parameters associated with the process in low ionic strength, D_2O, pH 7. The dissociation and formation rate constants are 0.65×10^4 sec^{-1} and 0.72×10^6 l. mol^{-1} sec^{-1}, respectively, at 41° and are consistent with the kinetic aspects of the helix–coil transition in related oligonucleotides determined from optical studies.

The helix–coil transition of the self-complementary duplex d-ApTpGpCpApT in H_2O solution has been investigated by monitoring the exchangeable Watson–Crick ring NH resonances by high-resolution proton nuclear magnetic resonance (NMR) spectroscopy. It was demonstrated that the hexanucleotide double helix opens in a sequential process from its ends (Patel, 1974a) (Figure 1A) and the thermodynamic parameters associated with the fraying of the individual base pairs were determined and interpreted (Patel and Hilbers, 1975). The studies were extended to describe the separation and characterization of the nucleation from the propagation reaction for helix formation and provide estimates of the kinetic parameters associated with the different steps (Hilbers and Patel, 1975). Further, a combination of proton and phosphorus NMR studies demonstrated that the phenoxazone ring of the peptide antibiotic actinomycin D intercalates between GC and CG central base pairs in double-helical d-ApTpGpCpApT in solution (Patel, 1974a,b). The above experiments were undertaken at low temperatures, conditions under which the double helix was predominantly intact, since at coil concentrations

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