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Electron Paramagnetic Resonance Study of Iodine-Induced Radicals of Benzo[*a*]pyrene and Other Polycyclic Hydrocarbons†

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ABSTRACT: Free radicals have been postulated previously as intermediates in the chemical linkage of the environmental carcinogen benzo[*a*]pyrene to nucleic acids when activated by iodine. Electron paramagnetic resonance studies indicate the presence of benzo[*a*]pyrene radicals in benzene, methanol and cyclohexane solution induced by iodine. These radicals are quenched by pyrimidine, purine, nucleosides, imidazole, and other nitrogenous compounds but not by alcohol, aldehyde, or water. These results strongly support the proposal that radicals of benzo[*a*]pyrene are involved in the chemical reaction between the hydrocarbon and nucleic acids in the presence of iodine. The electron paramagnetic resonance studies on the steady-state radical concentration of 14 polycyclic hydrocarbons formed in the presence of iodine indicate that, in general, the carcinogenic compounds such as benzo[*a*]pyrene, 7,12-dimethylbenzanthracene, 3-methylcholanthrene, etc., have a much higher concentration of radicals than the noncarcinogenic compounds such as benzo[*e*]pyrene, benzanthrene, pyrene, naphthacene, etc. There are one or two exceptions. The steady-state radical concentrations of these compounds do not correlate well with their ionization potentials, though the compounds having low ionization potentials do tend to yield higher concentration of radicals.

The scientific and public health significance of polycyclic hydrocarbon carcinogenesis has been generally well recognized (Bergman and Pullman, 1969). In the preceding papers of this series (Lesko *et al.*, 1969; Hoffmann *et al.*, 1970), our laboratory reported that B[*a*]P[1] and other carcinogenic polycyclic hydrocarbons react specifically with DNA and polynucleotides in the presence of I_2 in aqueous or aqueous ethanol systems. Under similar conditions noncarcinogenic analogs react with nucleic acid to a much lesser extent. It was postulated that radicals of B[*a*]P and other hydrocarbons may serve as reaction intermediates (Lesko *et al.*, 1969; Hoffmann *et al.*, 1970). Earlier, several investigators had proposed the radical cation of B[*a*]P as the intermediate in the reaction of B[*a*]P with pyridine or nucleic acid bases in a solid-phase system activated by I_2 vapor (Rochlitz, 1967; Wilk and Girke, 1969). In 1960, Szent-Györgyi *et al.* reported the existence of radicals of a number of compounds including B[*a*]P when activated by I_2.

We report in this paper electron paramagnetic resonance (epr) studies on formation and reactivities of radicals of B[*a*]P and related polycyclic hydrocarbons induced by I_2 in various organic solvent systems. The results strongly support the previous proposal that radicals of B[*a*]P and other hydrocarbons are formed in the presence of I_2, a mild oxidative system, and that these radicals participate in the chemical reaction with nucleic acid. The relationship of these findings

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[1] Abbreviations used are: B[*a*]P, benzo[*a*]pyrene; B[*e*]P, benzo[*e*]pyrene; MCA, 3-methylcholanthrene; DMBA, 7,12-dimethylbenz[*a*]anthracene; 5′-mmtr-(2′,3′)-ipr-guanosine, 5′-monomethoxytrityl-(2′,-3′)-isopropylideneguanosine; 5′-mmtr-(2′,3′)-ipr-adenosine, 5′-monomethoxytrityl-(2′,3′)-isopropylideneadenosine.

to the physical chemistry of the B[*a*]P oxidative process and to the initiation process involved in chemical carcinogenesis are discussed.

Experimental Section

Materials

The hydrocarbons used in these experiments were purchased from the following sources: pyrene, picene, dibenz[*a,c*]anthracene, dibenz[*a,h*]anthracene, B[*a*]P, anthracene, perylene, naphthacene from Aldrich Chemical Co., Milwaukee, Wis.; DMBA, MCA from Sigma Chemical Co., St. Louis, Mo.; naphthalene from Eastman Kodak Co., Rochester, N. Y.; benz[*a*]anthracene from Nutritional Biochemicals, Cleveland, Ohio; B[*e*]P from General Biochemicals, Chagrin Fall, Ohio; dibenzo[*a,j*]pyrene from K & K Laboratories, Plainview, N. Y. Purity of hydrocarbons was examined by thin-layer chromatography (Hoffmann *et al.*, 1970). Naphthacene showed the presence of two spots and was recrystallized from boiling benzene by addition of methanol.

Spectral grade benzene, methanol, and cyclohexane were purchased from Fisher Scientific Co., Pittsburgh, Pa.; molecular sieves were added to all solvents before use.

Other reagent grade chemicals used in these experiments came from the following sources: nitroimidazole, monomethoxytrityl chloride from Aldrich Chemical Co.; phenol (distilled before use), tetrahydrofuran from Fisher Scientific Co.; N^1-methylcytosine, N^9-methyladenine, N^3-methyluracil from Cyclo Chemical Co., Los Angeles, Calif.; pyridine, aniline, *N,N*-dimethylaniline from Baker Chemical Co.; purine from Sigma Chemical Co.; imidazole, *N,N*-dimethylformamide which was dried by distillation from phthalic anhydride onto molecular sieves, from Eastman Kodak Co.,; 2′,3′-isopropylideneadenosine, 2′,3′-isopropylideneguanosine, both checked for purity by thin-layer chromatography on Eastman silica gel sheets 6060, from Sigma Chemical Co.; silica gel (CC-7, 100–200 mesh) from Scientific Products, McGraw Park, Ill.; pyrimidine from Nutritional Biochemicals; 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl from Synvar, Palo Alto, Calif.

Synthesis of 5′-mmtr-(2′,3′)-ipr-adenosine. The protected nucleoside was synthesized using the modified procedures of Keuchler and Der Kosch (1966) and Lohrmann and Khorana (1964). Monomethoxytrityl chloride (1.20 g, 3.9 × 10^{-3} mol) dissolved in 20 ml of dry dimethylformamide was added to a dry sample of (2′,3′)-isopropylideneadenosine (1.00 g, 3.25 × 10^{-3} mol). An additional 20 ml of dimethylformamide was added and the reaction mixture was stirred at room temperature in the dark for 3 days. The reaction vessel was cooled to 0°, and 100 ml of water containing 2% ammonia was added slowly with stirring. After the flask warmed to room temperature, the precipitate was filtered and washed with 300 ml of anhydrous ether. Examination of the reaction mixture by thin-layer chromatography on silica gel sheets using chloroform–methanol (95:5, v/v, solvent C) showed several new minor products and one new major product. The crude product was dissolved in $CHCl_3$ and applied to a silica gel column (4.0 × 54 cm). The column was eluted with chloroform to remove the monomethoxytrityl alcohol. Subsequent elution with solvent C gave the desired product (1.05 g, 1.9 mmol) in pure form in 65% yield. The product was recrystallized from chloroform–hexane and precipitated as a white powder: mp 102–103° (lit. (Keuchler and Der Kosch, 1966) mp 102°); R_F 0.03 ether, 0.66 tetrahydrofuran–methanol (90:10, v/v, solvent B), 0.77 solvent C; nuclear magnetic resonance ($CDCl_3$) δ 7.68, 14 H, multiplet, aromatic C–H, δ 8.38 and 8.66, 2 H singlets, H-2 and H-8 of adenine; uv in methanol, λ_{max} 260 and 230 nm, λ_{min} 247 nm.

Synthesis of 5′-mmtr-(2′,3′)-ipr-guanosine (Kuechler and Der Kosch, 1966). Monomethoxytrityl chloride (1.50 g, 4.5 × 10^{-3} mol) was dissolved in 50 ml of dry dimethylformamide and 10 ml of dry pyridine was added to a dry sample of (2′,3′)-isopropylideneguanosine (1.00 g, 3.1 × 10^{-3} mol). An additional 20 ml of dimethylformamide was added and the reaction mixture was stirred at room temperature in the dark for 18 hr. The light brown solution was slowly poured onto ice water with stirring. The precipitate was filtered and washed with 300 ml of anhydrous ether. Examination of the remaining precipitate by thin-layer chromatography on silica gel sheets (solvent C) showed one new major product. The crude product was dissolved in chloroform and applied to a silica gel column (4 × 54 cm). Elution with approximately 600 ml of chloroform removed the monomethoxytrityl alcohol leaving the product at the top of the column. The silica gel containing the product was removed and reapplied to a fresh silica gel column (3 × 34 cm). Elution with solvent C yielded the desired compound (1.05 g) in 62% yield. The product was recrystallized from chloroform–hexane and precipitated as a white powder: mp 166–167° (lit. (Kuechler and Der Kosch, 1966) mp 165°); R_F 0.02 ether, 0.44 solvent B, 0.42 solvent C; nuclear magnetic resonance, $CDCl_3$ δ 1.85 and 2.16, 6 H, singlets, $C{-}CH_3$, δ 7.72, 14 H, multiplet, aromatic C–H; uv in methanol, λ_{max} 234 nm, shoulders at 250 and 271 nm.

Procedures

Electron Paramagnetic Resonance Studies on Radicals. Various polycyclic hydrocarbon solutions of known concentration were prepared by dissolving weighed solids in organic solvents (such as benzene), and the concentrations were verified by uv spectroscopy. Dilutions from the stock solution for uv studies were made in various solvents. The extinction coefficients (log ϵ) at the corresponding λ_{max} in various solvents for the polycyclic hydrocarbons used are: dibenzo[*a,j*]pyrene in benzene, 410 nm, 4.03; pyrene in methanol–ethanol, 333.5 nm, 4.71; perylene in methanol–ethanol, 368 nm, 3.68; dibenz[*a,h*]anthracene in dioxane, 350 nm, 4.16; dibenz[*a,c*]anthracene in ethanol, 349 nm, 3.45; B[*a*]P in ethanol, 366 nm, 4.44; B[*e*]P in ethanol, 366 nm, 3.80; benz[*a*]anthracene in ethanol, 341 nm, 3.87; anthracene in methanol–ethanol, 374.5 nm, 3.87.

Iodine solution was always freshly prepared by dissolving the weighed iodine in organic solvents (such as benzene) and was discarded after 2 days in solution. Equal volumes of iodine and hydrocarbon solutions were mixed at room temperature to yield the final concentration of both components in solution, and aliquots were withdrawn and frozen in liquid nitrogen after various time intervals.

The frozen samples were examined in a dual-cavity Varian E-9 epr spectrometer at approximately −30° for benzene and cyclohexane solutions and at −130° for methanol solutions. The radical concentrations were determined by simultaneously recording signals from both hydrocarbon solution and a standard solution prepared from weighed, stable, spin-label material (3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl) in the dual cavity of the spectrometer. The stability of the spin-label solution was evaluated by periodically checking against the Varian weak pitch standard. These signals were then both fed into a Fabritek 1074 signal averager where double integrations were performed and com-

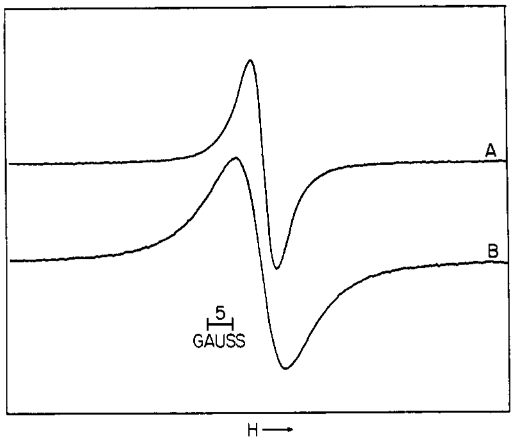


FIGURE 1: Epr spectra of B[*a*]P (A) and MCA (B) when mixed with I_2 in benzene and quick frozen in liquid nitrogen. Spectra taken at $-25°$ using hydrocarbon concentration of 5×10^{-4} M and I_2 concentration of 5×10^{-3} M. Modulation amplitude was 1.66; microwave power was 5 mW.

pared. The accuracy of the radical concentrations is about ±10%.

Absorption Spectra. Spectra of protected nucleosides and polycyclic hydrocarbons were obtained on either Cary 14 or 15 spectrophotometers.

Nuclear Magnetic Resonance Spectroscopy. Nuclear magnetic resonance data were obtained with a Varian HA-100 spectrometer with a temperature controlled probe. Chemical shifts are measured from an external Me_4Si capillary.

Melting Points. The protected nucleosides were melted in capillaries using a Thomas-Hoover apparatus; the values are uncorrected.

Ultraviolet Absorbance Studies on the Formation of Charge-Transfer Complex of B[a]P–I_2. All absorption spectra were measured in a Cary recording spectrophotometer, Model 14, with 1-cm quartz cells. Absorption of B[*a*]P (7.5×10^{-3} M) in cyclohexane starts rapidly at 430 nm toward lower wavelengths; absorption of I_2 in cyclohexane starts rapidly at 470 nm toward higher wavelengths; this leaves only a window at the region of 430–470 nm. The absorption of the B[*a*]P–I_2 complex in cyclohexane can be detected at this region (430–470 nm) when a solution of the complex was read against a reference solution having the same concentration of iodine. The absorption of the complex exhibited a broad shoulder starting at 470 nm and extending to 430 nm where the B[*a*]P begins to absorb strongly.

Results

Formation of B[a]P Radicals in Iodine Solution. Upon mixing a solution of B[*a*]P (10^{-3} M) and a solution of iodine (10^{-2} M) at room temperature, free radicals at $g = 2.003$ can be readily detected in the mixed solution in the frozen state (Figure 1). These radicals were not found in frozen solutions of I_2 or B[*a*]P alone, nor in mixed solution at temperatures above the freezing point of the solvent. The sharpness and height of the spectrum increased as the temperature was progressively lowered below the melting point. As shown in Figure 1, the spectra of both B[*a*]P and MCA radicals in iodine solution do not show any hyperfine structure in contrast to the spectrum of the cation radical of B[*a*]P formed in concentrated sulfuric acid (Nagata *et al.*, 1968; W. Caspary and P. Ts'o, unpublished data) and the spectra of the 6-oxy-B[*a*]P radical (Inomata and Nagata, 1972; R. Lorentzen and W. Caspary, unpublished results). Within the range of experimental fluctuation, the steady-state concentration of the B[*a*]P radical in benzene was reached rapidly (within 1 min) upon introduction of I_2 into the solution (Figure 2); the steady state in benzene was maintained for at least 2 hr, probably much longer. The maintainence of constant steady-state concentrations was also observed for other hydrocarbons in this study, such as B[*e*]P as shown in Figure 2.

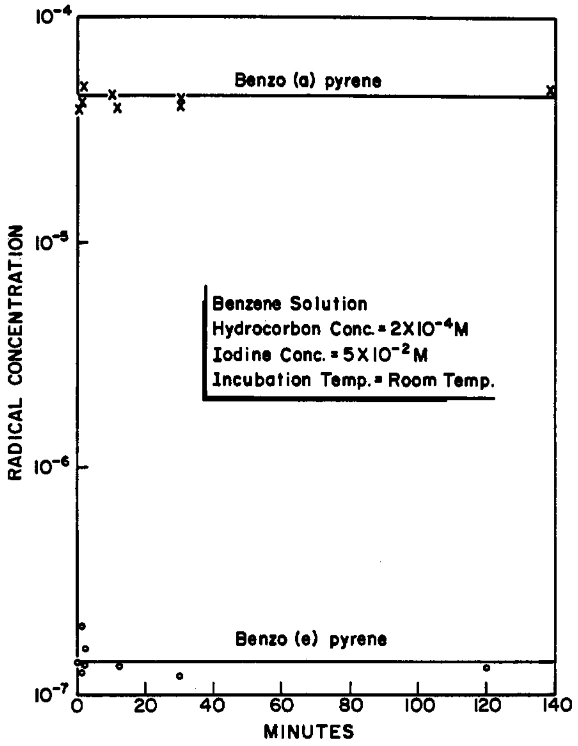


FIGURE 2: Comparison of steady-state radical concentration of the carcinogen B[*a*]P and noncarcinogen B[*e*]P. Radical concentration determined in frozen state ($-25°$).

Formation of radicals is critically dependent on the concentration of iodine and can be detected in solutions of benzene, cyclohexane, and methanol (Table I). The highest

TABLE I: Formation of B[*a*]P Radicals in Different Solvents and Iodine Concentrations.[a]

	Benzene ($-25°$)		Cyclohexane ($-25°$)		Methanol ($-120°$)	
I_2 Concn (M)	Radical[b] Concn (10^{-6} M)	% Radical[c]	Radical[b] Concn (10^{-6} M)	% Radical[c]	Radical[b] Concn (10^{-6} M)	% Radical[c]
2.5×10^{-1}	20	10				
5×10^{-2}	19	9.5			18	9
2.5×10^{-2}	20	10	20	10	18	9
5×10^{-3}	3	1.5	17	8.5	15	7.5
2.5×10^{-3}	0	0	19	9.5	6	3
5×10^{-4}	0	0	11	5.5	0	0
2.5×10^{-4}			6	3		
2×10^{-4}			7	3.5		

[a] B[*a*]P concentration at 2×10^{-4} M. [b] Accuracy ±10%. [c] (Radical concentration/B[*a*]P concentration) × 100.

TABLE II: Effects of Hydrocarbon Concentration and Iodine Concentration on the Per Cent of Radical Formed by B(*a*)P at Steady State as Induced by I_2 in Benzene Solution.[a]

I_2 Concn (M):	2.5×10^{-1}	5×10^{-2}	2.5×10^{-2}	5×10^{-3}
Hydrocarbon Concn (M)	$\frac{\text{Steady-State Radical Concn}}{\text{Initial Hydrocarbon Concn}} \times 100$			
5×10^{-4}	10	10	16.5	0.4
2×10^{-4}	10	9.5	10	1.5
5×10^{-5}	11	10	10	1.6

[a] The steady-state radical concentration was found to be the same when the solution, incubated at room temperature, was frozen within 1 min or within 2 hr after mixing. The solutions were always measured at frozen state.

TABLE III: Effect of Various Compounds on Quenching B[*a*]P Radical in Benzene Solution.[a]

Compound	Compound B[*a*]P	% Reduction in Radical Concn
Methanol	660	0
Isopropyl alcohol	350	0
Acetaldehyde	940	0
H_2O	1500	0
Removal of O_2 by flushing with N_2		0
5′-mmtr-(2′,3′)-ipr-guanosine	6.6	93
5′-mmtr-(2′,3′)-ipr-adenosine	8.3	99
Monomethoxytrityl alcohol	8.0	0

[a] Iodine concentration, 1.6×10^{-2} M; B[*a*]P concentration, 1.25×10^{-3} M. Samples were frozen 1 min after mixing.

concentration of radicals found in these solutions at the steady state was about 10% of the B[*a*]P concentration. This steady-state concentration of B[*a*]P radicals dropped to zero at 2.5×10^{-3} M I_2 in benzene as well as at 5×10^{-4} M I_2 in methanol, but still persisted in 2×10^{-4} M I_2 in cyclohexane (Table I). This appears to be a result of complex formation between I_2 and benzene (Benesi and Hildebrand, 1949), as well as different reaction rates of the radicals in methanol. Among these three solvents, cyclohexane is the most inert; thus, the I_2 concentration required for radical formation is the least. In Table II, it is shown that the concentration of radicals is proportional to the concentration of B[*a*]P; thus, the percentage of radicals remains constant within a tenfold change of B[*a*]P concentration.

Reactivity of B[*a*]*P Radicals.* Various substances were introduced into the B[*a*]P–I_2 system in benzene solution to study the reactivity of the B[*a*]P radicals. As shown in Table III, the radical concentration was not reduced by the introduction of methanol, isopropyl alcohol, acetaldehyde, and water, or by bubbling nitrogen gas through the system. The steady-state radical concentration in methanol did decrease slowly over a 2-hr period. The radical concentration, however, was drastically reduced upon introduction of adenosine and guanosine. These nucleosides were substituted at the sugar hydroxyl positions to increase their solubility in benzene; the 5′-substituted group, monomethoxytrityl alcohol, did not quench the radicals. These data indicate that the radicals are relatively inert toward alcohols, aldehyde, and water, but are reactive toward nucleosides. Following this lead, a variety of nitrogenous compounds were tested. In Table IV, it is shown that addition of many nitrogeneous compounds, such as imidazole, purine, pyrimidine, pyridine, aniline, and nucleosides, can quench the B[*a*]P radicals. Phenol did not have any effect. The extent of quenching is proportional to the nucleoside concentration; with a B[*a*]P concentration of 2×10^{-4} M and an I_2 concentration of 5×10^{-3} M, addition of 5′-mmtr-(2′,3′)-ipr-adenosine to the extent of 1–2 nucleoside residues per B[*a*]P molecule can still quench practically all the B[*a*]P radicals. Addition of the nucleoside at ratios of 0.5–0.05 nucleoside per B[*a*]P could only partially quench the radical, and the results became more variable. The quenching, however, was found also to be related to I_2 concentration. At an I_2 concentration of 5×10^{-2} M, the ratio of nucleoside per B[*a*]P needed for a complete quenching became much higher; the ratio could become as high as 100 for complete quenching to occur. At high concentrations of nucleosides the effectiveness of quenching was the same for either adenosine or guanosine. At low concentrations, where the results became much less accurate, adenosine appeared to be slightly more effective.

Formation of Radicals by Other Polycyclic Hydrocarbons in I_2 Solution. As shown in Figure 1, the carcinogenic hydrocarbon MCA also forms radicals in the presence of I_2. The noncarcinogenic hydrocarbon B[*e*]P produces much fewer radicals than B[*a*]P under similar conditions. The difference in the steady-state radical concentration produced by the carcinogenic B[*a*]P and that produced by the noncarcinogenic B[*e*]P was found to depend on the concentrations of I_2 and the hydrocarbon in the system. Table V shows that at high I_2 and high hydrocarbon concentrations, the ratio of B[*a*]P radical/B[*e*]P radical produced in the system was about 3 to 5; but under conditions of low I_2 concentration and low hydrocarbon concentration, the ratio can be as high as 100 in favor of B[*a*]P radicals. In other words, under conditions *favorable* to production of radicals, the differences in radical concentrations produced by these two hydrocarbons are only severalfold, while under *unfavorable* conditions (low I_2 concentration, etc.), the decrease in the production of radicals from the noncarcinogenic B[*e*]P is much larger than that from the carcinogenic B[*a*]P, resulting in a ratio that is 10- to 100-fold in favor of B[*a*]P.

In Table VI, the steady-state radical yields of 14 polycyclic hydrocarbons (2×10^{-4} M hydrocarbons, 5×10^{-2} M I_2) are tabulated together with their structures, carcinogenicities, and ionization potentials (Pullman *et al.*, 1969). The four powerful carcinogens, MCA, DMBA, B[*a*]P, and dibenzo-[*a,i*]pyrene, as well as the noncarcinogen perylene have large radical yields, while the noncarcinogenic compounds naphthalene, picene, B[*e*]P, pyrene, dibenz[*a,c*]anthracene, benz[*a*]-anthracene, anthracene, and naphthacene produce much less or no radicals. The weak carcinogen dibenz[*a,h*]anthracene also produced no radicals. These compounds were investigated in benzene solution, which is the best solvent for these hydrocarbons; addition of 1% water or about 20% methanol to the benzene system did not significantly change the results of those hydrocarbons tested. In benzene, changing the temperature of the frozen sample from −25 to −70° and evacuating the sample to remove dissolved oxygen have no effect on

TABLE IV: Quenching Effect of Various Compounds on B[*a*]P Radicals Activated by Iodine in Methanol Solution.[a]

Compound	% Reduction of Radical Concn 1 min after	% Reduction of Radical Concn 2 hr after
Imidazole	66	95
Nitroimidazole	31	100
Phenol	0	0
*N*¹-Methylcytosine	100	100
*N*³-Methyluracil	71	99
Purine	100	100
*N*⁹-Methyladenine	100	100
2′,3′-Isopropylideneadenosine	100	100
2′,3′-Isopropyleneguanosine	80	84
5′-mmtr-(2′,3′)-ipr-adenosine	100	100
5′-mmtr-(2′,3′)-ipr-guanosine	96	100
Aniline[b]	99	
N,*N*-Dimethylaniline[b]	99	
Pyridine[b]	99	
Pyrimidine[b]	99	

[a] The compound/B[*a*]P ratio was 10:1. B[*a*]P concentration, 2×10^{-4} M; I_2 concentration, 2.5×10^{-2} M. Four minutes after mixing the B[*a*]P and I_2, the compound was added and the solution was allowed to stand at room temperature for periods of 1 min and 2 hr before assaying at liquid nitrogen temperature of −130°. [b] The compound/B[*a*]P ratio was 8:1. B[*a*]P concentration, 1.25×10^{-3} M; I_2 concentration, 1.6×10^{-2} M.

TABLE V: Ratio of Steady-State Radical Concentration of B[*a*]P *vs.* That of the B[*e*]P under Various I_2 and Hydrocarbon Concentrations.

I_2 Concn (M):	5×10^{-2}	2.5×10^{-2}	5×10^{-3}
Hydrocarbon Concn (M)	Radical Concn of B[*a*]P / Radical Concn of B[*e*]P		
5×10^{-4}	5.1	4.6	100[a]
2×10^{-4}	5.0	3.0	20[a]
5×10^{-5}	10[a]	10[a]	10[a]

[a] The radical concentration of B[*e*]P is too low to be measured accurately.

the radical concentrations produced by B[*a*]P. B[*e*]P, MCA, perylene, and benzo[*a,j*]pyrene do appear to affect the steady-state radical concentration of DMBA. At the lower temperature and under evacuation, the radical concentration of DMBA can increase threefold. This study indicated that the DMBA radical is reactive and also sensitive to environment; currently more study on this subject is in progress.

The results in Table VI do not indicate a correlation between the steady-state radical concentrations and the ionization potentials. However, those compounds which yield a high concentration of radicals tend to have a low ionization potential (below 7.15 eV), though naphthacene, which has the lowest ionization potential (6.64–6.05 eV), is not a high-radical producer.

The radicals of the carcinogen MCA and the noncarcinogen perylene were found to be reactive toward substituted adenosine and guanosine, though the reactivity of perylene radicals appears to be less than those of B[*a*]P radicals and MCA radicals. When adenosine is added at an adenosine/hydrocarbon ratio of 0.5, the B[*a*]P radicals and MCA radicals are quenched by 50–60%, but the perylene radical concentration remains the same; with an adenosine/hydrocarbon ratio of 2, all the B[*a*]P radicals and MCA radicals are quenched completely while the perylene radicals are quenched about 80%. As for the radical of B[*e*]P, preliminary experiments indicated that this radical can also be quenched by a high concentration of substituted adenosine (ratio of 50:1)

Formation of Charge Transfer Complex between I_2 *and Benzo[a]pyrene.* Mulliken (1950, 1951) proposed that the intense characteristic absorption in a solution of I_2 and benzene is due to intermolecular charge transfer between a donor (benzene) and an acceptor (I_2). The work of Bhattacharya and Basu (1958) on the charge-transfer interactions between I_2 and polycyclic hydrocarbons (including naphthalene, anthracene, pyrene, etc.) confirmed Mulliken's theory on the relationship between increase in stability constants for interaction between an acceptor and donors with progressively lower ionization potentials. Since the ionization potential of B[*a*]P is quite low (Table VI), the stability constant of I_2–B[*a*]P transfer complex should be rather high, according to this established relationship. However, the work of Bhattacharya and Basu (1958) also indicated that the molar extinction coefficients of the complexes were found to diminish with increasing stability constants, contrary to Mulliken's theory. This turned out to be a serious handicap in our current investigation of the interaction between I_2 and B[*a*]P.

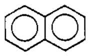
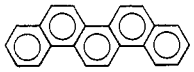
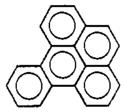
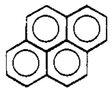
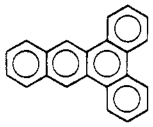
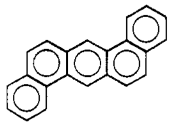
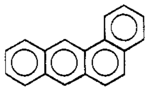
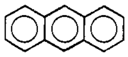
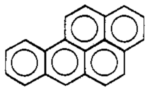
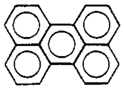
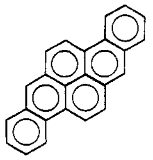
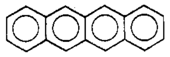
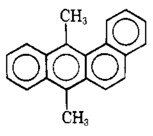
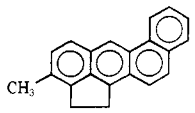
According to the theoretical prediction, the extinction coefficient of the charge-transfer complex between I_2 and B[*a*]P should be below 100. Consequently, this necessitated the use of high I_2 concentration and high B[*a*]P concentration in order to observe the complex in cyclohexane. A diffuse band with no characteristic maximum was observed between 435 and 470 nm, the only window available in a solution of cyclohexane containing 7.5×10^{-3} M I_2 and 7.4×10^{-3} M B[*a*]P. This diffuse band, which could not be observed in solution containing only I_2 or B[*a*]P (see Experimental Section), most likely is due to the formation of a charge-transfer complex. However, the measurements of absorbance (about 0.1–0.35 OD unit) of this band were not sufficiently precise, because of high background interference, to yield a linear relationship in an $[I_2]$/absorbance *vs.* 1/[B[*a*]P] plot for calculating the equilibrium constant for the I_2–B[*a*]P complex.

Discussion

These epr studies clearly demonstrate that B[*a*]P forms radicals in the presence of I_2. These radicals can be formed in benzene, cyclohexane, and methanol, and they are relatively inert toward alcohols, aldehydes, and water. On the other hand, though the reaction products have not yet been investigated, these radicals most likely react with the nitrogenous compounds, such as pyrimidine, purine, nucleosides, and imidazole, as indicated by the rapid quenching of the radicals.

In the previous papers (Lesko *et al.*, 1969; Hoffmann *et al.*, 1970) of this series, our laboratory reported extensive chemical reaction of the carcinogens B[*a*]P, MCA, and DMBA with DNA and polynucleotides when induced by I_2 in an aqueous system or aqueous ethanol system (ethanol added to increase the solubility of the hydrocarbons). These reactions are spe-

TABLE VI: Comparison of Ionization Potentials and Steady-State Radical Concentrations of Various Hydrocarbons after Mixing 5×10^{-2} M Iodine and 2×10^{-4} M Hydrocarbon.

Ionization Potentials (Spectroscopy)	Structure	Name	% of Hydrocarbons Found as Radical		
			Benzene	Benzene–Methanol Mix. (v/v, 4:1)	Benzene–Water Mix. (v/v, 99:1)
8.12–8.16		Naphthalene	0		
7.62–7.75		Picene	0		
7.60–7.73		Benzo[*e*]pyrene	2.5 ± 1.0		2.3
7.58–7.72		Pyrene	2.7		
7.43–7.6		Dibenz[*a,c*]-anthracene	0.2		
7.42–7.58		Dibenz[*a,h*]anthracene (weakly carcinogenic)	0		
7.35–7.53		Benz[*a*]anthracene	0.5		
7.23–7.43		Anthracene	0		
7.15–7.37		Benzo[*a*]pyrene (Carcinogenic)	11 ± 1	7.6	11
6.83–7.11		Perylene	7.4	11.9	8.7
6.75–7.04		Dibenz[*a,j*]pyrene (carcinogenic)	12.5	11.3	11.7
6.64–6.95		Naphthacene[a]	3.0		
		7,12-Dimethylbenz[*a*]anthracene[b] (carcinogenic)	9.0		3.5–7.0
		3-Methylcholanthrene (carcinogenic)	17	13.3	19.1

[a] Recrystallized. [b] It was found that the steady-state radical concentration of DMBA is sensitive to temperature and to atmosphere contrary to the B[*a*]P.

cific; under the same conditions, the noncarcinogens, B[*e*]P and benz[*a*]anthracene, react with the DNA to a much lesser extent (4–14-fold less). The data in this paper provide strong support to the hypothesis that the chemical reaction of these carcinogens with nucleic acid as induced by I_2 proceeds *via* a radical intermediate(s). The formation, stabilities and re-

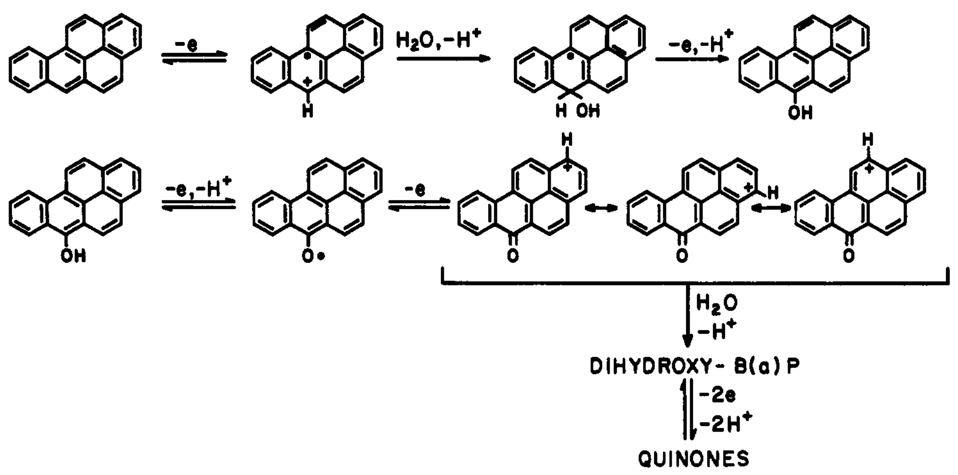


FIGURE 3: General reaction scheme for electrochemical oxidation of B[*a*]P proposed by Jeftic and Adams (1970).

activities of these hydrocarbon radicals indicate that these radicals can be the active intermediates of these chemical reactions.

As for the mechanism of the formation of B[*a*]P radicals, the general reaction scheme (Figure 3) proposed by Jeftic and Adams (1970) based on an electrochemical oxidation study on B[*a*]P appears to be applicable. In this scheme the oxidation of B[*a*]P can be conveniently thought to occur in two steps: the first step begins with formation of the radical cation, then the hydrated neutral radical, and finally results in 6-OH-B[*a*]P; the second step begins with the formation of a neutral oxo radical, which is then further oxidized to a carbonium ion, and finally ends with $(OH)_2$-B[*a*]P. The $(OH)_2$-B[*a*]P can be further oxidized to the quinone. All these radicals and the electrophilic carbonium ion species can potentially participate in chemical reactions with nucleic acids.

The presence of all these radical species in dynamic equilibrium may be a reason why no hyperfine structure in the epr signal can be observed. However, other explanations are possible. 6-OH-B[*a*]P spontaneously forms the oxy radical, and we have observed this radical at room temperature and at −30° (frozen) both in the presence and absence of I_2. Although the solution spectra both with and without I_2 manifest the characteristic hyperfine structure of this radical (Nagata *et al.*, 1968), freezing these samples reduced the multiplet to a singlet (W. Caspary, unpublished results). While the effect of I_2 on the hyperfine structure of the B[*a*]P radical is unknown, the presence of an I_2–B[*a*]P charge-transfer complex is predicted theoretically and is indicated experimentally, though this complex has not been extensively investigated in the present study. Such an interaction could presumably cause the observed loss of hyperfine structure. Nevertheless, while I_2 has no effect on the hyperfine structure of the oxo radical of 6-OH-B[*a*]P *in solution*, in the *frozen state* the hyperfine structure is reduced to a singlet. It should be remembered that all the B[*a*]P radicals formed in the presence of I_2 were observed in the *frozen state*. Since the amplitude of these I_2-induced radicals increases with decreasing temperature and since the epr signal disappears upon melting, it appears that the lack of signal at room temperature is due to relaxation effects, the relaxation time being so short that Heisenberg's uncertainty principle plays a dominant role. The observation of the oxo radical of 6-OH-B[*a*]P at room temperature would argue that this radical is not an important contributor to the radical signal observed by reacting I_2 with B[*a*]P, although it does not argue against the Jeftic and Adams scheme since only small amounts may be present in the pseudo-equilibrium mixture.

It is not unexpected that the steady-state concentrations of radicals produced by the 12 hydrocarbons so far investigated are not correlated with the ionization potentials of these compounds (Table VI). Since the radical concentration measured is governed by the balance between rate of formation and rate of decay, a compound, such as naphthacene which has the lowest ionization potential, may not yield the highest steady-state concentration of radicals if the radical also has a high rate of decay. On the whole, however, the hydrocarbons which have low ionization potential and therefore greater ease in surrendering an electron tend to yield a higher steady-state radical concentration in the I_2 oxidative system. One may expect, however, that if the initiation process in hydrocarbon carcinogenesis is related to the chemical reaction with these radicals, then the hydrocarbon which yields a high steady-state concentration of radicals will have carcinogenic activity, especially when this radical is chemically reactive to biologically active substances (such as nitrogenous compounds) but is stable in aqueous medium. By and large, this expectation is verified though not with perfect correlation among the fourteen compounds so far investigated. Among the exceptions is the compound perylene which is not a carcinogen but yields a relatively high steady-state concentration of radicals. This radical may be, however, less reactive toward the nucleosides. The other exception is the weak carcinogen dibenz[*a,h*]anthracene which does not yield any radicals. These exceptions indicate the need for further investigation and possibly the limitation of this model system of chemical oxidation of hydrocarbons. Hopefully, this study may lead to a convenient and reliable procedure for screening for the carcinogenic potential of polycyclic hydrocarbons.

Formation of 6-OH-B[*a*]P and its radical after incubation of B[*a*]P in liver homogenates with TPN has been reported by Nagata *et al.* (1968). Our laboratory has also reported the reaction of chemically synthesized 6-OH-B[*a*]P with nucleic acid (Lesko *et al.*, 1971; R. Lorentzen and P. Ts'o, 1971, unpublished data) as well as spontaneous formation of radicals from 6-OH-B[*a*]P (Caspary *et al.*, 1973). These findings will be the subject of the next paper in the series.

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Ultracentrifugal Study of Polydisperse and Paucidisperse Biological Systems Using Capillary Microcells†

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ABSTRACT: An ultracentrifugal technique based in the transport method to study polydisperse and/or paucidisperse biological systems is reported. The sedimentation cells used were either capillary tubes holding about 4 μl of dilute solutions or small cellulose tubes of 0.8-ml capacity. The methodology was used in connection with a Model L Beckman Spinco ultracentrifuge and an SW 50.1 swinging bucket rotor. Some of the concepts and procedures applied were presented in a previous publication (Pita, J. C., and Müller, F. J. (1972), *Anal. Biochem. 47*, 395). Three modes of approach were studied: (a) successive aliquot extractions along the tube length; (b) analysis of integral concentrations in the centripetal segment of the cell; and (c) centrifugations at different speed settings. The distribution function, $g(s)$, is obtained as a second derivative curve of the experimental concentrations found across the boundary gradient region. The plateau region was also studied and recognized to yield, when the experimental data are adequately plotted, straight line plots, with slope close to unity. The theory is easily extended to the case of paucidisperse systems. Boundary spreading due to diffusion is distinguished from polydispersity spreading. Several biological specimens, human serum albumin, preparations of a proteoglycan complex and its subunit, as well as a hypothetical trigonometric example, were used to analyze the possibilities of the technique. Results are found in good agreement with those obtained with conventional ultracentrifugal methods as reported in the literature.

The study of physical properties related to centrifugal heterogeneity shown by systems composed of a monomer in the presence of one or more polymers (paucidisperse systems) or those constituted by a polydisperse or continuous distribution of molecular weights is important for the correct understanding of biological mechanisms.

A previous publication (Pita and Müller, 1972) described a technique, based in the transport method, that permits the evaluation of sedimentation coefficients using only a few nanograms of biological principles. Purity of the sample was not required as long as the contaminants did not interfere with the analytical assay used. However, only single, monocomponent, solutions were considered in detail. It is the purpose of the current report to describe a methodology which, applying some of the concepts and experimental procedures included in the above-mentioned publication, allows the ultracentrifugal study of paucidisperse and polydisperse biological systems as well.

The exact shape and extent of the sedimenting boundary were, advantageously, of no relevance in the determination of sedimentation coefficients by the transport method (Pita and Müller, 1972). All measurements to that purpose were conducted in the plateau region, the boundary itself being considered as an ideal sharp demarcation without loss of mathematical rigor. In the present work, however, it is precisely the size, shape, and extent of the boundary that has to be studied, since according to Baldwin (1954) "the boundary gradient

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