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Electron Spin Resonance Study of ^{17}O-Enriched Oxybenzo[a]pyrene Radical

AMY S. RISPIN AND HIDEO KON

Laboratory of Chemical Physics, National Institute of Arthritis, Metabolism, and Digestive Diseases

DANIEL W. NEBERT

Developmental Pharmacology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20014

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SUMMARY

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Benzo[a]pyrene was incubated *in vitro* with cofactors and liver microsomes from rats previously treated with β-naphthoflavone, and the polycyclic hydrocarbon and its metabolites were extracted with a benzene–acetone mixture. Following evaporation of the solvent, the residue was dissolved in toluene or in ethanol–potassium phosphate buffer and examined by room-temperature electron spin resonance spectroscopy. Contrary to reports from other laboratories, the ESR signal respresenting an oxybenzo[a]pyrene free radical is initially very small or undetectable. Under various experimental conditions, we could not measure directly the free radical signal in aliquots from the microsomal incubation mixture. The signal rapidly increases in intensity during examination by ESR, and reaches a maximum in 2–3 hr, and lasts for at least 12 hr. The appearance and growth of the signal do not occur when the solvent used for ESR analysis is deoxygenated. Incubations with benzo[a]pyrene, cofactors, and microsomes under an atmosphere of $^{17}O_2$ demonstrate that the source of oxygen in the radical is atmospheric and the reaction is mediated by cytochrome P-448 or P-450, but that the free radical we observed occurs nonenzymatically, presumably via abstraction of a hydrogen atom (1-electron air oxidation) by molecular oxygen in the solvent used for ESR analysis. Whether this reaction is important in the binding of benzo[a]pyrene metabolites to DNA and in the etiology of chemical carcinogenesis remains to be determined.

INTRODUCTION

Numerous studies of chemical carcinogenesis have focused on benzo[a]pyrene and its metabolites (1–10). Of prime importance is the question of the identity of active intermediates formed during the metabolism of benzo[a]pyrene. By means of electron spin resonance, a free radical signal is observed when benzo[a]pyrene is incubated with a rat liver homogenate and then is extracted with benzene (1, 3, 4, 9, 10). Those authors characterized the free radical as 6-oxybenzo[a]pyrene radical. Generation of this free radical is considered to be enzymatic, and radical binding with DNA is implicated (1, 3, 4).

The object of this study is to determine whether formation of the free radical from

benzo[a]pyrene is cytochrome P-450-dependent, to demonstrate that the radical is an oxy radical, and to show that the source of oxygen in the radical is molecular oxygen. To accomplish these ends we incubated benzo[a]pyrene with rat liver microsomes under an atmosphere of $^{17}O_2$. The experimental design which we used is similar to that used by Nagata and co-workers, except that microsomes instead of a postnuclear liver fraction were used. The appearance of ^{17}O hyperfine structure in the ESR spectrum of the free radical is definitive evidence of enzymatic incorporation of oxygen from molecular oxygen. We found that the incubation enzymatically produced an oxygen-containing metabolite of benzo[a]pyrene, a precursor of the free radical. The oxybenzo[a]pyrene free radical which we observed, however, is formed largely, if not completely, by air oxidation of the precursor after extraction of the metabolites from the incubation mixture which contained the microsomes and cofactors.

EXPERIMENTAL PROCEDURE

Materials. The microsomes were prepared from livers of 80-g male Sprague-Dawley rats with a slight modification of the method used in our laboratory (11). The rats were treated 48 hr prior to death with β-naphthoflavone[1] (Aldrich Chemical Company) in corn oil, 100 mg/kg of body weight.

Benzo[a]pyrene (Aldrich Chemical Company) was dissolved in benzene and passed through a dry alumina column. The benzene was then evaporated under vacuum. Purified samples which gave no free radical signal were used. NADH and NADPH were obtained from Sigma Chemical Company, and enriched $^{17}O_2$ gas (^{17}O, 94.84 atom %, and ^{18}O, 0.89%), from Miles Laboratories.

[1] β-Naphthoflavone functions about the same as 3-methylcholanthrene as an inducer of cytochrome P-448 (or P_1-450) and numerous microsomal drug-metabolizing enzyme activities in genetically responsive animals (12, 13). The flavone appears not to be carcinogenic. For these reasons, β-naphthoflavone instead of 3-methylcholanthrene is often used in our laboratories.

Methods. The incubation was performed with 2 ml of microsomal suspension containing approximately 75 nmoles of cytochrome P-450 in 30% glycerol–0.25 M potassium phosphate buffer, pH 7.25; 60 mg of NADPH, 60 mg of NADH, and 200 nmoles of $MgCl_2$ in 20 ml of Tris-chloride buffer (pH 7.2); and 20 μmoles of benzo[a]pyrene, added in 1 ml of acetone. In certain experiments 6 mg each of NADPH and NADH, instead of 60 mg, were used in the usual incubation procedure. Prior to incubation, all components were kept at or below 4°. Incubations were carried out in subdued light under air with stirring at various temperatures (2.5°, 21.5°, and 37°). After incubation, benzo[a]pyrene metabolites were extracted twice into 50 ml of benzene–acetone (25:1, v/v). Solvent was removed from the extract with a rotary evaporator. The residue of metabolites was subsequently dissolved in dry toluene or ethanol–0.25 M potassium phosphate buffer, pH 7.2 (1:1, v/v). The ESR spectrum was observed with a Varian V-4502 X-band spectrometer at room temperature.

Benzo[a]pyrene was incubated with microsomes and $^{17}O_2$ in the following manner. The benzo[a]pyrene solution in acetone and the incubation mixture (microsomes, cofactors, $MgCl_2$, and buffer) were each degassed separately, the benzo[a]pyrene solution by repeated cycles of freezing, pumping, and thawing, and the incubation mixture by pumping and flushing with pure dry nitrogen gas. The two solutions were mixed under positive nitrogen pressure to prevent contact with air. The nitrogen pressure was then adjusted and $^{17}O_2$ was added so that nitrogen and oxygen were present in a 4:1 ratio of partial pressures totaling 1 atm. Incubation took place with stirring for 10 min at 21.5°. The system was brought below 4° and degassed thoroughly for 20 min to remove unreacted $^{17}O_2$. The system was then opened to the air and extracted as usual.

RESULTS

Formation of free radical from precursor. Benzo[a]pyrene was incubated in a manner similar to that described (1, 3, 4), the metabolites were extracted, and the

solvent was evaporated. Immediately after the residue had been dissolved in toluene, the ESR signal was very small or, in several experiments, undetectable. A signal appeared after 10–15 min and grew in a sigmoid fashion (whether or not the sample was exposed to light), reaching a maximum in 2–3 hr. The free radical signal, which appeared identical with that observed by others (1, 3, 4, 9, 10), lasted for about 12 hr and then gradually decayed.

It should be emphasized that, technically, considerable time is required between the end of the microsomal incubation and the ultimate measurements of the redissolved metabolite residue in organic solvent in the ESR spectrometer cavity, because of the extraction procedure itself, rotary evaporation of the extraction solvent, etc. The time usually required by us for these procedures was about 30 min. Whatever free radicals occur during the microsomal incubation (and their rate of decay) could not be followed directly by ESR analysis because of the low concentration of the radical in the incubation mixture and the small volume of aqueous solution able to fit in the flat cell for room-temperature ESR analysis. Such formation and/or disappearance of free radicals during the microsomal incubation until the time the metabolite residue is redissolved and examined in the ESR cavity remains, therefore, entirely speculative. During the microsomal incubation aqueous aliquots were examined in the flat cell at room temperature and were also quickly frozen and examined at 77°K; all such attempts to measure a free radical signal were unsuccessful.

When the solution of the radical in toluene was frozen at 77°K, no signal was observed; upon thawing to room temperature, however, the signal reappeared immediately. The disappearance and rapid reappearance of this signal suggest that the radicals associate reversibly to form a diamagnetic species at 77°K (2). To eliminate the possibility that air oxidation of benzo[*a*]pyrene might account for the development of the ESR signal, we dissolved purified benzo[*a*]pyrene in toluene; it gave no ESR signal over an 18-hr period.

Dependence of free radical on oxygen in solvent. Formation of the free radical in the sample cell is dependent upon oxygen in the organic solvent used for ESR analysis. To demonstrate this, we divided one sample of benzo[*a*]pyrene metabolites dissolved in toluene into two parts. In one sample, which had been degassed immediately after toluene was added to the residue, no signal appeared upon prolonged standing. The control sample, which was left open to air, gave no signal at first. The signal appeared and grew with time in the usual manner. When this latter sample was degassed 60 min later, the signal was still observed but decayed during observation. When the sample was re-exposed to air, the signal again increased as more free radicals formed.

Formation of the radical occurs in aqueous as well as organic media. When the extract of benzo[*a*]pyrene metabolites had been dissolved in ethanol–potassium phosphate buffer, growth of the free radical signal was similar to that observed in toluene solution. The rate of increase in signal height was not as pronounced, however, and, upon degassing, the free radical decayed more rapidly. This finding is consistent with the recent reports of Ts'o and co-workers (9, 10).

Our observations are consistent with the hypothesis that a precursor is formed enzymatically during incubation and is then converted to a free radical by air oxidation. This point was confirmed by various controls. Cofactors (NADPH more so than NADH) were found to be essential for enzymatic formation of the precursor. The rate of increase in the ESR signal height was similar whether 6 or 60 mg of NADPH and NADH had been used in the microsomal incubation. Incubation of benzo[*a*]pyrene with cofactors and boiled microsomes gave no free radical, even upon prolonged observation. Furthermore, no radical was formed when benzo[*a*]pyrene was incubated anaerobically with the microsomal preparation under nitrogen.

Dependence of free radical formation on time and temperature of microsomal incubation. Figure 1 shows that the relative signal intensity was actually greater when the microsomal incubation had been performed for 10 min at 26.5° rather than

36.5°. Further experiments showed that the maximal signal development occurred when the prior incubation had been carried out at 21.5°, but the difference was only about 30% greater than that observed when the incubation had been carried out at 36.5°. Approximately 20% of the maximal signal intensity developed even when the prior incubation had been performed at 2.5°. In each experiment, little or no ESR signal was detected initially when the toluene was added to the residue, but within several minutes the signal grew quite rapidly with time.

As had been noted by others (1, 3, 4, 9, 10), we found that the ultimate radical yield increases with prior incubation time to a maximum and then diminishes (Fig. 2). If the microsomes and cofactors had been incubated at 37° with benzo[a]pyrene for 30 min or longer, the amount of ESR signal was very small or not detectable at all. This result indicates that the precursor is converted by the microsomes to an end product (e.g., glucuronide conjugate) which does not become a radical by air oxidation in the toluene.

Our data therefore suggest that the rate of radical precursor formation, relative to the rate(s) of further metabolism of this precursor, is maximal at 21.5°. Moreover, incubation times longer than 20 min also favor the rate(s) of further metabolism of this precursor, compared with the rate of radical precursor formation.[2]

Incubation of microsomes, cofactors, and benzo[a]pyrene with $^{17}O_2$. To demonstrate that molecular oxygen was essential to the formation of the precursor of the oxybenzo[a]pyrene radical, we incubated benzo[a]pyrene with microsomes and cofactors under $^{17}O_2$. As before, when dissolved in toluene and in the presence of ordinary air, the sample gave no signal at first. When the signal intensity had attained sufficient size for careful analysis, the sample was degassed to improve the signal resolution (Fig. 3). The spectrum of the radical containing ^{17}O (Fig. 3B) was almost twice as wide as the spectrum of

[2] For further discussion of the possible fate of benzo[a]pyrene and its metabolites, see the recent reviews (14-16) on this subject.

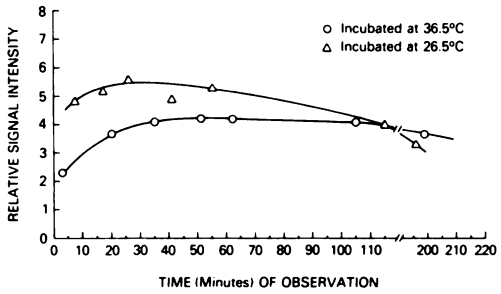


FIG. 1. *Variation of relative signal intensities of oxybenzo[a] pyrene free radical as a function of time after addition of toluene to the residue in open air*

Two temperatures (36.5° and 26.5°) at which microsomes and cofactors were incubated with benzo[a]pyrene are shown.

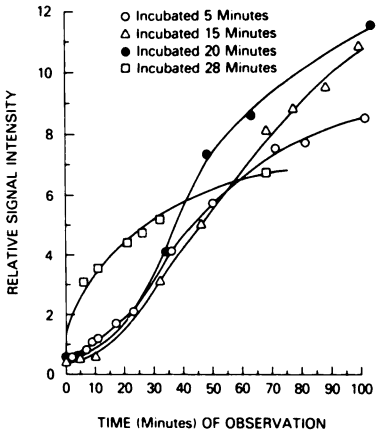


FIG. 2. *Effect of incubation time at 37° on rate of development of signal intensities of oxybenzo[a]pyrene free radical*

Relative intensities are given as a function of observation time. Specimens incubated for 5, 15, and 20 min, which were measured immediately after extraction, evaporation, and dissolution in toluene, show sigmoid growth. The specimen incubated for 28 min was observed after a delay of 45 min and thus does not show the complete sigmoidal curve.

the radical containing ^{16}O (Fig. 3A) and showed six prominent peaks due to hyperfine interaction with the ^{17}O nucleus (nuclear spin = 5/2). The hyperfine splitting constant due to the ^{17}O nucleus was 4.75 G. Computer simulation demonstrated that the ^{17}O spectrum is a 6-fold superposition of ^{16}O signals, each displaced by 4.75 G (Fig. 3C). The presence of six equal ^{17}O peaks indicates that ^{17}O was incorporated only once into the ring. It is unlikely that

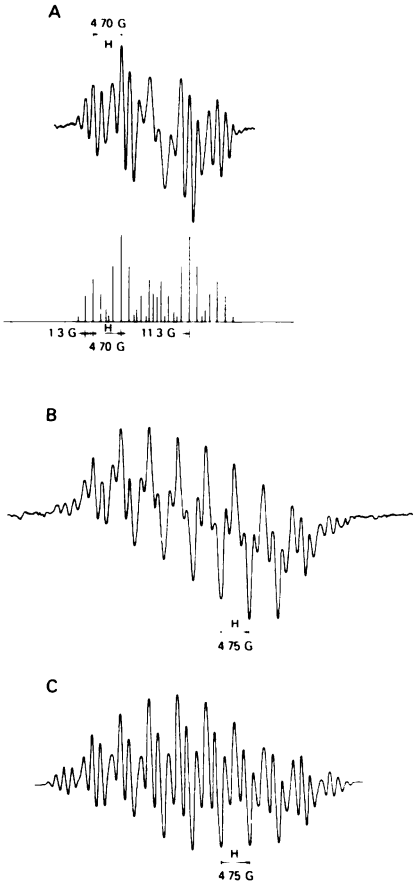


FIG. 3. *ESR signal of oxybenzo[a]pyrene free radical dissolved in toluene.*

A. Spectrum and schematic diagram of free radical produced by incubating benzo[a]pyrene with rat liver microsomes under $^{16}O_2$. B. Spectrum of free radical produced by incubating benzo[a]pyrene and rat liver microsomes under $^{17}O_2$. C. Computer simulation of ^{17}O radical spectrum.

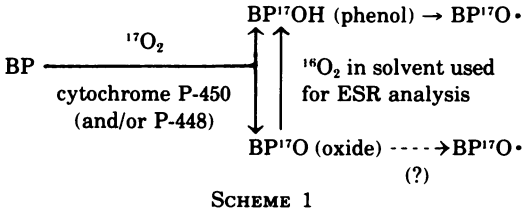
other oxygen atoms exist in this molecular species, unless such oxygen atoms occur in regions of vanishing spin density.

Appropriate controls showed that formation of the precursor and the radical was not affected by degassing either before or after incubation, so long as $^{17}O_2$-containing gas had been present during the microsomal incubation. Also, the fact that no signal was detected when O_2 was removed and replaced by nitrogen prior to incubation demonstrated the completeness of our degassing.

Although the increase in signal height depended on the presence of air in the solvent used for ESR analysis, the [^{17}O]benzo[a]pyrene radical spectrum showed no evidence of any incorporation of ^{16}O into the radical. Only the ^{17}O hyperfine structure increased in proportion to the signal growth. This finding indicates conclusively that the function of air in the solvent is a 1-electron oxidation of the precursor.

DISCUSSION

In this report we have confirmed the general observations from the laboratories of Nagata (1, 4) and Ts'o (3, 9, 10) that, following incubation of benzo[a]pyrene with rat liver fractions, extraction of metabolites, and evaporation of the extraction solvent, a free radical signal can be observed in the organic solvent used to dissolve the residue for ESR analysis. However, we also found that this signal develops in the organic solvent in the presence of oxygen. By using an atmosphere of $^{17}O_2$ during incubation with the microsomes, we conclude that the oxygen is incorporated enzymatically via cytochrome P-450 (or P-448) into benzo[a]pyrene during the incubation with microsomes and cofactors. By comparing oxygen-containing with nitrogen-containing solvents used for ESR analysis, we conclude further that the function of oxygen in the solvent is a 1-electron oxidation of the enzymatically formed precursor to form the free radical detected by ESR. These conclusions are summarized in Scheme 1, where BP stands



SCHEME 1

for benzo[a]pyrene. Formation of phenol by rearrangement of the oxide is known to occur, and direct insertion of a hydroxyl group mediated by cytochromes P-450 and P-448 has been postulated (see refs. 14–16 for discussions of this subject). There exists the possibility (17) that the K-region 4,5-oxide of benzo[a]pyrene could rearrange to form 3-hydroxybenzo[a]pyrene,

and benzo[a]pyrene-3,6-quinone. The 6-hydroxybenzo[a]pyrene derivative has recently been shown (9, 10) to be air-oxidized nonenzymatically to the free radical in the presence of a rat liver homogenate. Whether other phenolic derivatives or oxide intermediates can be similarly air-oxidized remains to be demonstrated. However, the formation of all three known quinones of benzo[a]pyrene—1,6-, 3,6-, and 6,12-quinone—*via* the 6-hydroxybenzo[a]-pyrene intermediate (9, 10) does appear to be the most likely mechanism for quinone formation.

The ^{16}O spectrum which we observed is clearly a homogeneous free radical species. Our ^{17}O spectral evidence that only 1 oxygen atom is incorporated into the oxybenzo[a]pyrene free radical eliminates any possibility that the signal represents a semiquinone radical. This finding confirms Nagata's assertion (4) and the recent work of Ts'o and co-workers (9, 10) that the signal is indeed an oxybenzo[a]pyrene radical.

In this report there is no definitive proof as to the presence or absence of free radicals actually generated in the incubation mixture. We found no signals from aliquots withdrawn during the incubation and quickly extracted and examined either at room temperature or frozen. Under these conditions the signal level might be too low for detection, or the radicals might dimerize to form a diamagnetic species. It is conceivable, however, that the free radical may be generated in the microsomal incubation mixture if the precursor and molecular oxygen are both present. Ts'o and co-workers have found (9, 10) that 6-hydroxybenzo[a]pyrene is air-oxidized to the free radical. Since the rate of 6-hydroxybenzo[a]pyrene disappearance is unchanged when cofactors are eliminated or when boiled rat liver homogenate is used, Ts'o *et al.* concluded that the oxidation is nonenzymatic. Our observation of rapid disappearance of the radical in the absence of air, together with the finding that radicals can be observed in the toluene extract in the presence of air for 12 hr or more, implies that the extract contains a large amount of enzymatically formed precursors.

Our observations that the ESR signal is initially very small or undetectable are somewhat at variance with findings from other laboratories.[3] However, the incubation system those workers used was total rat liver homogenate or a postnuclear supernatant fraction (after one low-speed centrifugation), whereas we incubated the benzo[a]pyrene with NADPH, NADH, and rat liver microsomes. The presence of mitochondria and cytosol may affect in some manner the steady-state level of the precursor and/or its conversion to the free radical in the presence of molecular oxygen. Therefore minor differences in results may be due to these different incubation conditions and time factors involved during the extraction. The ^{16}O signal which we observed, however, is identical with that observed in the laboratories of Nagata and Ts'o. And the intensity of the ESR signal which we observed represents, in large part if not altogether, the free radical population formed by air oxidation of the enzymatically generated precursor by oxygen in the solvent used for ESR analysis.

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