Specificity of chemiluminescence in the metabolism of benzo[a]pyrene to its carcinogenic diol epoxide

(microsomal chemiluminescence/chemiluminescence quantum yields/dioxetane of benzo[a]pyrene)

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ABSTRACT The metabolism of 7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene results primarily in the ultimate carcinogenic metabolite of benzo[a]pyrene, 7,8-dihydroxy-9,10-oxo-7,8,9,10-tetrahydrobenzo[a]pyrene and to a lesser extent 7,8-dihydroxy-9,10-dioxetane-7,8,9,10-tetrahydrobenzo[a]pyrene, from which chemiluminescence is observed. This specific microsomal chemiluminescence has been used to establish that the rate-limiting reaction in the metabolism of benzo[a]pyrene to the bay region diol epoxide is the production of the 7,8-diol. The microsome-mediated chemiluminescence of the parent benzo[a]pyrene is therefore an indicator of the activity of the specific sequence of metabolic reactions leading to the ultimate carcinogenic metabolite.

The detoxication reactions of the carcinogenic polycyclic aromatic hydrocarbon benzo[a]pyrene (BP) have been studied extensively in order to explain the fortuitous production of highly reactive electrophilic metabolites that are mutagenic and carcinogenic. There appear to be common structural features of carcinogenic polycyclic aromatic hydrocarbons such that a specific sequence of metabolism results in bay region diol epoxides that are mutagenic (1-6), bind to nucleic acids (7-14), produce transformation in cell culture (15, 16), and are carcinogenic *in vivo* (17-23). On the basis of these structural features we have predicted and observed a specific chemiluminescence (CL) concomitant with the microsomal metabolism of BP and other carcinogenic polycyclic aromatic hydrocarbons by induced rat liver microsomal extracts (24-26). We have reported that the CL observed is the result of an intermolecular electron exchange decomposition of the dioxetane of 7,8-diol-BP, produced during the further metabolism of 7,8-diol-BP to the carcinogenic epoxide of 7,8-diol-BP (27). We now report the microsomal CL quantum yields of BP and 17 BP derivatives, and the comparison between induced and noninduced rat liver microsomal systems of the temporal kinetics and concentration kinetics of the production of metabolites and their CL intensities. It is proposed that 7,8-diol-BP is a metabolic intermediate that is formed in rate-limiting steps and that is further metabolized into *both* its carcinogenic 9,10-epoxide and its chemiluminescent 9,10-dioxetane. Therefore, it is proposed that the microsomal CL of BP can be used as a sensitive indicator for the carcinogenic 9,10-epoxide and ultimately as a sensitive measure of the extent of this specific carcinogenic pathway, even in the presence of the much more common metabolic pathways leading to noncarcinogenic metabolites.

MATERIALS AND METHODS

NADPH, bovine serum albumin, and Tris were obtained from Sigma. 3-Methylcholanthrene, BP, and 9,10-dihydrobenzo[a]pyrene-7(8H)-one were from Aldrich. ($\pm$)-7,9-Dihydroxy-7,8-

dihydro-BP (7,8-diol-BP), 4,5-diol-BP, 9,10-diol-BP, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydro-BP (diol epoxide), and the 12 monohydroxy derivatives of BP (1-OH-BP, etc.) were obtained from the chemical repository of the National Institutes of Health. 9,10-Dihydro-BP and 7,8-dihydroxy-7,8,9,10-tetrahydro-BP were synthesized from 9,10-dihydrobenzo[a]pyrene-7(8H)-one (28, 29). 7,8,9,10-Tetrahydroxy-7,8,9,10-tetrahydro-BP was obtained by solvolysis of the diol epoxide (30). The hydrocarbons were purified by high-performance liquid chromatography (HPLC) and concentrations were determined by ultraviolet absorption.

Liver microsomes were prepared from control and 3-methylcholanthrene-induced Long-Evans rats (25). Protein concentrations were determined by a modified Lowry procedure with bovine serum albumin as the standard (31).

3-OH-BP production was assayed by fluorescence of alkali-soluble products (32) and by HPLC.

For the HPLC quantitation of 3-OH-BP, 7,8-diol-BP, and 7,8,9,10-tetrol-BP metabolites, the reaction mixture composition and sample preparation were the same as those of Selkirk *et al.* (33). The metabolites were separated by HPLC on a 4.2 mm $\times$ 25 cm ODS-2 column (Whatman), eluted with a 60-95% gradient of methanol in water at a flow rate of 1 ml min⁻¹. Quantitation was by peak height.

The rate of 7,8-diol-BP metabolism was measured by following the decrease in fluorescence intensity at 407 nm (367-nm excitation).

CL intensities were measured in a light-collecting geometry similar to that used for liquid scintillation counting by single photon counting techniques. The absolute photon efficiency was determined with the luminol chemiluminescent reaction (34). For the microsome-mediated CL the reaction mixture contained Tris-HCl, pH 7.6 (50 mM), MgCl₂ (3 mM), microsomes, hydrocarbon (in 150 μ l of methanol), and NADPH (0.5 mM) in a final volume of 3 ml. The reactants were incubated at 37°C for 10 min before the reaction was initiated with NADPH.

RESULTS AND DISCUSSION

The chemiluminescence quantum yields (ϕ_{CL}) for the microsome-mediated CL of BP and 17 derivatives of BP were determined at low substrate concentrations such that the total light was approximately proportional to substrate concentration. The ϕ_{CL} is equal to the chemical yield (ϕ_{chem}) for the formation of a dioxetane during the epoxidase reaction, multiplied by the fraction of dioxetanes that participate in an intermolecular elec-

Abbreviations: BP, benzo[a]pyrene; CL, chemiluminescence; ϕ_{CL} , chemiluminescence yield; HPLC, high-performance liquid chromatography; diol epoxide, 7,8-dihydroxy-9,10-oxo-7,8,9,10-tetrahydro-BP; tetrol, 7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydro-BP; 1-OH-BP, 1-hydroxy-BP; 7,8-diol-BP, 7,8-dihydroxy-7,8-dihydro-BP. The other phenols and diols are similarly designated.

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Table 1. Microsomal CL quantum yields (ϕ_{CL})

Hydrocarbon	Relative ϕ_{CL} *	Microsome-activated mutagenicity†
High yield		
7,8-Diol-BP	1.0	+++
BP	0.3	++
1-OH-BP	0.4	+
3-OH-BP	0.4	+
4-OH-BP	0.3	—
6-OH-BP	0.3	+
Low yield		
2-OH-BP	0.03	+
5-OH-BP	0.05	—
7-OH-BP	0.004	—
8-OH-BP	0.01	—
9-OH-BP	0.004	+
10-OH-BP	0.02	—
11-OH-BP	0.01	—
12-OH-BP	0.03	+
4,5-Diol-BP	0.004	—
9,10-Diol-BP	0.03	—

The microsomal reaction with 3-methylcholanthrene-induced microsomes at 1 mg ml⁻¹ was run for various concentrations of each substrate. The intensity versus time curves were integrated to determine the total light emission. For the concentration region where the total light was proportional to the concentration, ϕ_{CL} was calculated by dividing the total light by the total number of substrate molecules initially added.

* ϕ_{CL} of 7,8-diol-BP = 7.0×10^{-9} photons per molecule.

† From ref. 35.

tron exchange to form a radical anion–radical cation pair ($\phi_{\pm}$) that recombines to leave the product in an electronically excited state (exciplex), multiplied by the fluorescence quantum yield (ϕ_f) for the exciplex product:

$$\phi_{CL} = \phi_{chem} \times \phi_{\pm} \times \phi_f. \quad [1]$$

At the present time the three factors in Eq. 1 cannot be separated.

The CL quantum yields for BP and 15 BP monophenol and diol derivatives are shown in Table 1. Except for substitutions affecting the carbon atoms 2 and 5, the values of ϕ_{CL} for substitutions on the pyrene moiety are the same as those for BP. Substitutions on the benzo ring that would preclude the formation of the 7,8-diol-9,10-dioxetane have values of ϕ_{CL} between 0.1 and 0.004 relative to ϕ_{CL} for the 7,8-diol-BP metabolite. Substitutions on the 11 and 12 positions also result in extremely low values of ϕ_{CL} , consistent with inhibition of type III geometry (25). In particular, saturation at the 9,10 position, which precludes 9,10-dioxetane formation, results in a lower ϕ_{CL} relative to 7,8-diol-BP as shown by the following ratios:

$$\frac{\phi_{CL}(9,10\text{-diol-BP})}{\phi_{CL}(7,8\text{-diol-BP})} = 0.03 \quad [2]$$

$$\frac{\phi_{CL}(9,10\text{-dihydro-BP})}{\phi_{CL}(7,8\text{-diol-BP})} = 0.1 \quad [3]$$

$$\frac{\phi_{CL}(7,8\text{-diol-9,10-dihydro-BP})}{\phi_{CL}(7,8\text{-diol-BP})} = 0.1. \quad [4]$$

The ϕ_{CL} of the 9,10-diol-BP fits the category of low-yield hydrocarbons described in Table 1. The ϕ_{CL} values of 9,10-dihydro-BP and 7,8-diol-9,10-dihydro-BP, while significantly less

than the value of 7,8-diol-BP, appear to be intermediate between the high- and low-yield groups.

It appears that the regiospecificity for production of a chemiluminescent diol-dioxetane on the benzo ring (ϕ_{chem}) is much greater for the 7,8-diol than for the 9,10-diol. These CL data are consistent with the reports that saturation of the 7,8 bond of BP directs metabolism to the 9,10 bond (36, 37), whereas saturation of the 9,10 bond directs metabolism to the pyrene portion of the molecule (38). From the ratios 3 and 4 a minimum of 90% of the CL of 7,8-diol-BP can be ascribed to dioxetane formation at the 9,10 bond, because saturation of this bond (i.e., 9,10-dihydro) precludes dioxetane production. As shown in Fig. 1, the microsomal CL maximal intensities of 7,8-diol-BP and 7,8-diol-9,10-dihydro-BP substrates show saturation kinetics, whereas for BP and 9,10-dihydro-BP the microsomal CL intensities are inhibited at higher concentrations. This same concentration inhibition of CL intensity occurs for 1-OH-BP, 3-OH-BP, and 4-OH-BP. In these latter cases if remetabolism of a primary metabolite were required for CL, additional substrate would compete for the limiting enzyme. It has been reported that at high substrate concentrations initial metabolites are predominantly formed, whereas at low substrate concentrations the primary metabolites are remetabolized (39).

For any given microsomal extract, the ratio

$$\frac{CL_{max}(BP) [\text{photons s}^{-1} \text{ nmol}^{-1} (\text{mg protein})^{-1}]}{\text{rate of 3-OH-BP production from BP} [\text{pmol min}^{-1} (\text{mg protein})^{-1}]} \quad [5]$$

is a constant (24). For induced and noninduced microsomal extracts the maximum CL intensities and the rates of metabolite production gave the ratios:

FIG. 1. Concentration dependence for the maximal microsomal CL intensity of: (A) 7,8-diol-BP (●) and 7,8-diol-9,10-dihydro-BP (■), and (B) BP (●) and 9,10-dihydro-BP (■). The hydrocarbons were added to 3 mg of 3-methylcholanthrene-induced rat liver microsomes in 3 ml of Tris-HCl buffer, pH 7.4, containing 3 mM MgCl₂ at the concentration indicated. The reactants were incubated at 37°C for 10 min before the addition of NADPH (0.5 mM).

$$\frac{[\text{CL (7,8-diol-BP)}]_{\text{induced}}}{[\text{CL (7,8-diol-BP)}]_{\text{noninduced}}} = \frac{\left[\frac{\text{rate of 3-OH-BP production from BP}}{\text{rate of 7,8-diol-BP production}} \right]_{\text{induced}}}{\left[\frac{\text{rate of 3-OH-BP production from BP}}{\text{rate of 7,8-diol-BP production}} \right]_{\text{noninduced}}} = 4, \quad [6]$$

$$\frac{[\text{rate of 7,8-diol-BP production}]_{\text{induced}}}{[\text{rate of 7,8-diol-BP production}]_{\text{noninduced}}} > 10, \quad [7]$$

and

$$\frac{[\text{CL (BP)}]_{\text{induced}}}{[\text{CL (BP)}]_{\text{noninduced}}} \approx 16. \quad [8]$$

It appears that the enzyme(s) that produce the 3-OH-BP metabolite from BP and the CL of 7,8-diol-BP (Eq. 6) are induced to different degrees than the enzyme(s) that produce the 7,8-diol-BP metabolite (Eq. 7), which is also assumed to be the precursor to the CL of BP (Eq. 8).

When 7,8-diol-BP is used as a substrate there is a very rapid increase in CL to a maximal intensity (26, 27). However, under the same conditions with BP as a substrate the rise in CL is much slower (27), indicating that the production of the 7,8-diol-BP metabolite is the rate-limiting reaction in the microsomal CL of BP.

The apparent first-order rate constant for the metabolism of 7,8-diol-BP as measured by the disappearance of 7,8-diol-BP was 0.014 s^{-1} (0.2 mg ml^{-1} of induced microsomes, $2 \mu\text{M}$ 7,8-diol-BP), whereas the apparent first-order rate constant for the decay of the CL was 0.006 s^{-1} , indicating that the 7,8-diol-9,10-dioxetane-BP has a lifetime significantly greater than 1 min.

The relative mutagenic activity of these metabolites, microsome-activated in the *Salmonella typhimurium* test system, are shown in the last column of Table 1. We assume that the ϕ_{CL} measured is due primarily to the production of a diol at the 7,8 bond as the result of metabolism of the substrates listed in Table 1. Because the continued metabolism of the 7,8-diol precursor will result in the predominant formation of the diol epoxide as well as a small fraction of dioxetanes, the ϕ_{CL} should be correlated with mutagenicity of the substrates. It is not proposed that the observed CL represents a significant production of a new species of alkylating metabolite. The CL appears to be a sensitive optical technique for measuring the metabolism of the distal bay region diol of BP to the bay region diol epoxide.

The lack of reported mutagenicity of 4-OH-BP appears to be an exception to the proposed correlation. The 9-OH-BP cannot be metabolized to the diol epoxide; its alkylation to DNA is at the K-region bond (40). It is conceivable that the mutagenicity of the 2- and 12-monophenols also may not involve a bay region diol epoxide.

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