Benzo[*a*]pyrene Diol Epoxides: Mechanism of Enzymatic Formation and Optically Active Intermediates

Abstract. *Studies of the mechanism of benzo[*a*]pyrene metabolism to reactive diol epoxides and of their disposition indicate that the metabolic intermediates of the activation pathways, 7,8-epoxide and* trans*-7,8-diol, as well as the two stereoisomeric diol epoxides are all optically active. Benzo[*a*]pyrene is converted to optically active 9,10-epoxides of (−)*trans*-7,8-diol by three enzymatic steps: (i) stereospecific oxygenation at the 7,8 double bond of benzo[*a*]pyrene by the mixed-function oxidases to essentially a single enantiomer of 7,8-epoxide, (ii) hydration of the 7,8-epoxide by epoxide hydratase to an optically pure (−)*trans*-7,8-diol, and (iii) stereoselective oxygenation by the mixed-function oxidases at the 9,10 double bond of the (−) trans-7,8-diol to optically active* r-7,t-8*-dihydroxy-*t*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene and optically active* r-7,t-8*-dihydroxy-*c*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene in a ratio of approximately 10 to 1.*

Benzo[*a*]pyrene (BP), the most common polycyclic aromatic hydrocarbon in our environment (*1*), requires metabolic activation by the microsomal enzymes to exert several biological effects including toxicity, mutagenicity, and carcinogenicity (*2*). Benzo[*a*]pyrene was found to be metabolized to three diols (*3–7*), four phenols (*8*), and three quinones (*4–7*). These metabolites were resolved by high-pressure liquid chromatography (HPLC) (*4–6*). However, the more polar metabolites were eluted as one chromatographic peak by previous methods (*5–9*). Recently a more efficient column enabled us to analyze the polar metabolites (*10, 11*) in more detail.

Each of the three diols of BP can exist as either a *cis* or a *trans* isomer. Each of the configurational isomers can exist as a (+) or a (−) enantiomer or as a racemic mixture. Optically active diols of naphthalene, phenanthrene, and anthracene were found to exist as the *trans* isomer in mammals (*12–16*) but the *cis* isomer in bacteria (*16*). We recently reported that the 7,8-diol formed metabolically from BP by rat liver microsomes is the *trans* isomer (*17*) and is optically active (*10, 18*).

The *trans*-7,8-diol is more mutagenic than BP and 11 other BP derivatives when tested in cultured Chinese hamster V79 cells cocultivated with polycyclic hydrocarbon–metabolizing normal cells (*11*). Two stereoisomeric 9,10-epoxides of BP *trans*-7,8-diol have been synthesized (*19–21*) and their stereochemistries have been elucidated (*20, 21*). The *r*-7,*t*-8-dihydroxy-*c*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (diol epoxide II) (*22*) was found to be more mutagenic in strains TA 98 and TA 100 of *Salmonella typhimurium* and in cultured Chinese hamster V79 cells than BP 4,5-epoxide (*23*). The *r*-7,-*t*-8-dihydroxy-*t*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (diol epoxide I) (*22*), however, was found to be more mutagenic in mammalian cells than diol epoxide II, 4,5-epoxide, and 13 other BP derivatives tested including all the known BP metabolites (*11*). The exceptionally high mutagenic activity of diol epoxide I in mammalian cells has been confirmed (*24, 25*).

trans-7,8-Diol formed by incubation of BP with liver microsomes from rats treated with 3-methylcholanthrene was extracted and isolated by HPLC (*10*). The optical rotation of the metabolically formed *trans*-7,8-diol was compared to that of the optically pure (−)*trans*-7,8-diol resolved by HPLC (*26*). The results (Table 1) indicate that the metabolically formed *trans*-7,8-diol is almost exclusively the (−) enantiomer. A further improvement of the recently developed HPLC method (*26*) has made it possible to resolve completely the di-(−)menthoxyacetates of (+) and (−) *trans*-7,8-diols (Fig. 1). The di-(−)menthoxyacetate of the *trans*-7,8-diol formed metabolically from BP is found to elute only with the resolved di-(−)menthoxyacetate of the (−)*trans*-7,8-diol (Fig. 1). The results thus establish that the metabolically formed *trans*-7,8-diol is the (−) enantiomer.

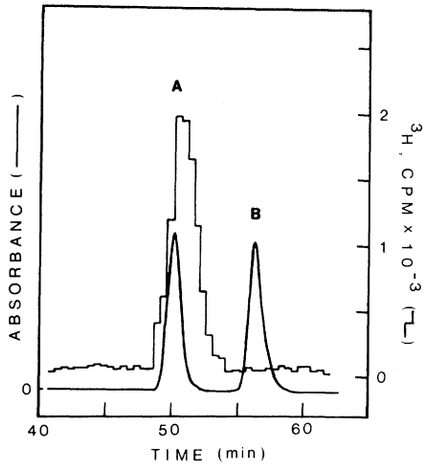

Fig. 1. Separation of di-(−)menthoxyacetates of BP (−)*trans*-7,8-diol (peak A) and (+)*trans*-7,8-diol (peak B) by HPLC. The metabolically formed [^{3}H]*trans*-7,8-diol (~ 0.1 μg) and nonradioactive synthetic (+)*trans*-7,8-diol (50 μg) in 0.1 ml of pyridine were reacted with (−)menthoxyacetyl chloride (10 mg) for 16 hours at room temperature, ~24°C (*40*). Water (1 ml) was added to the mixture, which was then extracted with three 2-ml portions of diethyl ether and benzene (1:1 by volume). The organic extract was evaporated to dryness with a gentle stream of nitrogen. The sample was dissolved in 50 μl of methylene chloride just before HPLC analysis. The HPLC was carried out on a DuPont model 830 liquid chromatograph fitted with a DuPont Zorbax SIL (silica adsorbent) column (25 cm long and 6.2 mm inner diameter). The column was first equilibrated with methylene chloride, and the sample was eluted with methylene chloride at a flow rate of 2 ml/min at room temperature. The identities of peak A and peak B have been established (*26*). Abbreviation: CPM, counts per minute.

Epoxide hydratase has been reported to be a relatively nonspecific enzyme (*27*). Enzymatic hydration of racemic naphthaiene 1,2-epoxide (*15*) yields the corresponding *trans*-1,2-diol and more than 90 percent of the oxygen from the attack of solvent water is introduced at C_2 (*28*). In the metabolism of naphthalene with mouse liver homogenates, the oxygen was found to be derived from molecular oxygen at C_1 of the *trans*-1,2-diol (*14*). The detailed mechanism of the enzymatic hydration of racemic BP 7,8-epoxide has not been previously reported.

Table 1 shows that racemic BP 7,8-epoxide is converted by partially purified epoxide hydratase to *trans*-7,8-diol containing 86 percent of the (−) enantiomer in addition to the rearrangement product BP 7-phenol. The results indicate that epoxide hydratase is stereoselective in the hydration of the 7,8-epoxide which is the specific precursor of the (−)*trans*-7,8-diol. If epoxide hydratase were nonspecific toward racemic 7,8-epoxide, a racemic *trans*-7,8-diol would be formed.

The stereoselective nature of epoxide hydratase (Table 1) is indicated by the observation that approximately 6.1 times as much (−)*trans*-7,8-diol as (+)*trans*-7,8-diol is formed from the racemic 7,8-epoxide. With this ratio of enzymatic hydration products, an enantiomeric 7,8-epoxide mixture enriched 19 to 1 in favor of the precursor of the (−)*trans*-7,8-diol could result in a *trans*-7,8-diol containing 99.1 percent of the (−) enantiomer. Thus, if the mixed-function oxidases converting BP to the 7,8-epoxide and the subsequent epoxide hydratase catalyzing the formation of *trans*-7,8-diol are both highly stereoselective, the product of the two enzymatic reactions would be close to optically pure.

An optically pure (−)*trans*-7,8-diol could be formed from BP by one of several mechanisms: (i) nonspecific formation of a racemic 7,8-epoxide, followed by ste-

reospecific hydration of only one of the enantiomers to optically pure (−)*trans*-7,8-diol; (ii) stereoselective formation of 7,8-epoxide with an excess of one enantiomer, followed by a further stereoselective formation of the latter to yield (−)*trans*-7,8-diol of high purity; or (iii) stereospecific formation of a single enantiomer of the 7,8-epoxide, followed by stereospecific hydration to an optically pure (−)*trans*-7,8-diol. The first mechanism would require that the modes of enzymatic hydration of both 7,8-epoxide enantiomers be different—that is, the water molecule attacks one of the enantiomers at C_7 and the other at C_8—or that only one of the enantiomers is subjected to enzymatic hydration. This is unlikely, and the results in Table 1 argue against this mechanism since a racemic 7,8-epoxide yields a *trans*-7,8-diol containing 86 percent of the (−) enantiomer. Table 1 further shows that the mechanism must be either (ii) or (iii)—that is, that one of the enantiomers of the 7,8-epoxide is formed either predominantly or exclusively and that the planar BP molecule is highly stereoselectively or stereospecifically oxygenated at one side of the 7,8 double bond. Further studies of the optically pure (−)*trans*-7,8-diol enzymatically formed from BP with rat liver microsomes under oxygen-18 gas (*17*) indicated that the oxygen of the C_7 OH was exclusively derived from molecular oxygen. Thus BP is stereospecifically oxygenated at one side of the 7,8 double bond to form a single enantiomer of the 7,8-epoxide intermediate.

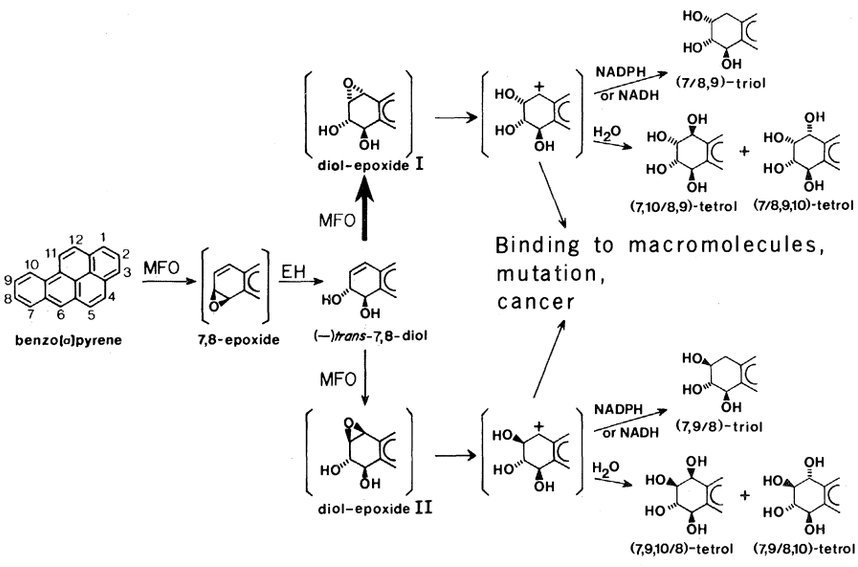

Fig. 2. Mechanism of metabolic formation and disposition of benzo[*a*]pyrene diol epoxides. Abbreviations: *MFO*, mixed-function oxidases; *EH*, epoxide hydratase. Triangles and dotted lines indicate that the substituents are toward and away from the viewer, respectively (*18, 41*).

Table 1. Optical rotations of the *trans*-7,8-diol resolved by HPLC and enzymatically formed from BP and racemic BP 7,8-epoxide. The optical rotations were measured on a Perkin-Elmer model 241-MC polarimeter; $[\alpha]^{25}_{400}$ is the specific rotation at 400 nm and 25°C. The HPLC-resolved (−)*trans*-7,8-dihydrobenzo[*a*]pyrene-7,8-di-(−)menthoxyacetate is optically pure and free of (+)*trans*-7,8-dihydrobenzo[*a*]pyrene-7,8-di-(−)menthoxyacetate (Fig. 1) (*26*). Thus the (−)*trans*-7,8-diol obtained by methanolysis (in CH_3ONa-CH_3OH-tetrahydrofuran at 60°C for 10 minutes) of its diester is optically pure.

Source of *trans*-7,8-diol	*trans*-7,8-Diol		(−) Enantiomer (%)
	Concentration in methanol (mg/ml)	$[\alpha]^{25}_{400}$	
Resolved by HPLC*	0.19	−3730 ± 160	100
BP metabolism†	0.25	−3482 ± 62	97
Racemic BP 7,8-epoxide‡	0.49	−2701 ± 62	86

*The racemic *trans*-7,8-diol was reacted with (–)menthoxyacetyl chloride, and the diastereoisomers were resolved by HPLC (*26*). †The *trans*-7,8-diol was prepared by incubation of BP with liver microsomes from 3-methylcholanthrene–treated male Sprague-Dawley rats and isolated by HPLC (*10*). ‡A 2-liter reaction mixture, containing 8.4 mg of racemic BP 7,8-epoxide, 6.4 mg of protein equivalent of partially purified epoxide hydratase from livers of phenobarbital-treated male Sprague-Dawley rats [free of aryl hydrocarbon hydroxylase activity and purified as described in (*39*)] and 0.1 mole of tris-HCl, *p*H 7.4, was incubated under nitrogen at 37°C. The racemic BP 7,8-epoxide was dissolved in 50 ml of tetrahydrofuran, and 10 ml was added to the reaction mixture at time zero and every 60 minutes thereafter. At the end of 5 hours, the reaction mixture was extracted with two 2-liter volumes of ethyl acetate and the organic phase was dehydrated with anhydrous magnesium sulfate and evaporated to dryness under reduced pressure. The *trans*-7,8-diol (final yield, 1.31 mg) and 7-hydroxybenzo[*a*]pyrene (final yield, 2.44 mg) were isolated by HPLC.

The (−)*trans*-7,8-diol has been shown (*10*) to be further metabolized stereoselectively to a major diol epoxide I and a minor diol epoxide II in a ratio of approximately 10 to 1. Since the (−)*trans*-7,8-diol is optically pure, the hydroxyl groups are fixed in one of two possible configurations. Therefore each of the metabolically formed diol epoxides is a single enantiomer. The diol epoxides formed metabolically are unstable in aqueous media and each is hydrolyzed to a pair of tetrahydroxytetrahydrobenzo[*a*]pyrenes (tetrols) (*10, 11*) and nonenzymatically reduced to a trihydroxypentahydrobenzo[*a*]pyrene (triol) by reduced nicotinamide-adenine dinucleotide or reduced nicotinamide-adenine dinucleotide phosphate (*10, 29*). The tetrols and triols derived from the metabolically formed optically active diol epoxides are therefore all predicted to be optically active. Diol epoxide I is hydrolyzed *trans*-stereoselectively to a major (7,10/8,9)-tetrol and a minor (7/8,9,10)-tetrol, but diol epoxide II is hydrolyzed *cis*-stereoselectively to a major (7,9,10/8)-tetrol and a minor (7,9/8,10)-tetrol (*18, 30*). Both diol epoxides I and II are hydrolyzed through carbonium ion intermediates at C_{10} (*29–30*).

The enzymatic mechanism of BP metabolism to reactive diol epoxides and their disposition are depicted in Fig. 2. Recent studies (*31–35*) have indicated that diol epoxides are involved in binding to DNA and RNA in vitro and to mammalian cells in culture. Diol epoxide I has been demonstrated to be the major species formed metabolically (*10, 11, 34, 35*) and bound to DNA (*36, 37*) and RNA (*34*) and the most mutagenic BP metabolite in cultured mammalian cells (*11, 24, 25*). Racemic BP 7,8-epoxide has been found to be carcinogenic on mouse skin (*38*). In view of these findings, the major metabolically formed and optically active diol epoxide I may be the predominant carcinogenic form of benzo[*a*]pyrene.

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17. Under HPLC conditions, which separate BP *cis*- and *trans*-7,8-diols, we found that the metabolically formed 7,8-diol is a *trans* isomer. Mass spectral analysis indicated that the *trans*-7,8-diol formed enzymatically from BP under $^{18}O_2$ contained ^{18}O. Acid treatment of this ^{18}O-containing *trans*-7,8-diol yielded 7-^{18}OH-BP (97 percent) and 8-OH-BP (3 percent), which were separated by HPLC. An ^{18}O-containing *trans*-7,8-diol was also obtained by incubating racemic BP 7,8-epoxide in $^{18}OH_2$ with partially purified epoxide hydratase. On acid treatment of this ^{18}O-containing *trans*-7,8-diol, the ^{18}O was found exclusively in 8-OH-BP by mass spectral analysis. (S. K. Yang, P. P. Roller, H. V. Gelboin, in preparation).
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Phyllotaxis in *Xanthium* Shoots Altered by Gibberellic Acid

Abstract. *Gibberellic acid treatment of vegetative* Xanthium *shoots induced a change in phyllotaxis and almost doubled the rate of leaf production. Phyllotaxis in control plants displayed a 2,3 contact parastichy pattern; that of the treated plants could be approximated with a 3,5 pattern. Thus, the* Xanthium *apex switched to a new mode of growth and a higher order of phyllotactic leaf arrangement not seen in untreated plants. It may be inferred from these experiments that gibberellic acid plays a role in determining the site of leaf initiation.*

Single application of gibberellic acid (GA) produced a striking change in the shape of leaves and accelerated the rate of leaf initiation in vegetatively grown plants of *Xanthium pennsylvanicum* by a factor of 1.8 (*1*). The stimulation of leaf production coincided with enhancement of cell division and enlargement of the apical dome (*2*). We provide evidence here that the hormone treatment also leads to a striking change in phyllotaxis, from what might be designated as a 2,3 pattern of leaf arrangement to a 3,5 pattern, where the index numbers 2, 3, and 5 designate sets of contact parastichies (*3*). To our knowledge, this is a first report and data presentation indicating that prolonged treatment with GA causes marked changes in phyllotaxis.

Plants of *X. pennsylvanicum* were grown in a walk-in type growth chamber where the incandescent light of about 700 foot-candles (~ 7500 lu/m^2) cycled to give 18 hours of illumination per day, to prevent flowering. Other plants were grown in a greenhouse, supplemented with incandescent light to provide 16 hours of illumination. A lanolin paste containing GA (0.7 mg, 75 percent K salt, in 100 mg of paste per plant) was applied once to each of a number of randomly chosen plants of nearly uniform plastochron age, and comparable plants of similar plastochron age, not treated with the paste, were designated as controls. Leaves on each shoot were numbered in order of their appearance distally from the cotyledons, and records were kept of leaf numbers for each shoot. The morphological ages of plants and leaves were estimated by the plastochron index (*PI*) and the leaf plastochron index (*LPI*) (*4*). Apical buds were fixed overnight in a 3:1 mixture of ethanol and acetic acid at various times after treatment. After embedding in Tissue-Prep, 8-μm serial sections were cut transversely to the shoot axis, through the apical region, and stained with Feulgen reagent and fast green, or Delafield hematoxylin and fast green. Camera lucida drawings were made, at a magnification of 63×, of sections just below the apical meristem, chosen to include the youngest visible primordium.

Determination of leaf arrangement was carried out by methods of Richards (*3*) and Maksymowych and Erickson (*5*). The latter method is based on measurements of divergence angle and determination of the plastochron ratio (a), which involves measurements of the chord lengths between three successive leaf primordia in cross sections of apical shoots (Fig. 1). The relative plastochron rate of radial displacement is ln a. Values of the parameters α and ln a characterize various patterns with orthogonal and contact parastichies (Table 1). Details of the analysis are presented elsewhere (*5*). Richards's method (*3*) is based on measurements of radial distances of successive leaf primordia from the stem apex and estimation of the divergence angle. To designate a pattern of leaf arrangement, he proposed the use of the phyllotaxis index. This index is designated to have approximately the value of 1 for a 1,2 pattern of orthogonal parastichies, 2 for 2,3, 3 for 3,5, and so on, for an assumed divergence angle $\alpha = 137.51°$. Both methods are extensions and modifications of Van Iterson's (*6*) models of phyllotaxis.

The effect of the GA treatment on the organization of the shoot apex was similar in all of the treated plants. This is illustrated in Fig. 1. There is an increase in the number of leaf primordia on the treated shoot, and they appear to be more closely spaced than those of the control. The apparent larger size of the apical meristem in Fig. 1A is in part due to the level at which the section was cut,