Vol. 82, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1978 Pages 518-525

THE MICROSOMAL METABOLISM OF BENZO(a)PYRENE PHENOLS

Jorge Capdevila, Ronald W. Estabrook, and Russell A. Prough
Department of Biochemistry
The University of Texas Health Science Center at Dallas
5323 Harry Hines Blvd., Dallas, Texas 75235

Received April 6,1978

Analysis of repetitive scan difference spectra of incubation mixtures containing rat liver microsomes, 3- or 9-hydroxybenzo(a)pyrene, oxygen, and NADPH shows the formation of products with absorbance in the 400-450 nm region. Based on the chromatographic retention time, absorbance, and fluorescence spectra, the two major products of 9-hydroxybenzo(a)pyrene metabolism may be diphenols. The existence of spectral intermediates which resemble phenols rather than quinones during the steady-state metabolism of 3-hydroxybenzo(a)pyrene strongly indicates that either the major product is a diphenol which slowly oxidizes to yield 3,6-quinone and/or that an active quinone reductase exists in liver microsomes.

The mechanism of further metabolism of B(a)P[1]-phenols and the formation of B(a)P-quinones remains largely unexplained. Nagata et al.(1) and Ts'o et al. (2,3) have documented the oxygen-dependent conversion of the unstable 6-hydroxy-B(a)P to quinones by a reaction which involves free radical intermediates. Wiebel (4) and Yang et al. (5) have presented evidence that 3-hydroxy-B(a)P is oxidatively metabolized by liver microsomes to products which include the 3,6-quinone as the major metabolite. Other studies on the further metabolism of the 3- and 9-phenol have shown that both compounds yield products which covalently bind to cellular nucleophiles, such as DNA (6,7). The present report describes the application of difference spectrophotometry coupled with HPLC techniques to monitor the steady-state metabolism of 3- and 9-hydroxy-B(a)P by liver microsomes from 3-methylcholanthrene pretreated rats. It is suggested that during the steady-state of the reaction polyoxygenated products, such as diphenols, may result from the NADPH- and oxygen-dependent metabolism catalyzed by a microsomal oxygenase.

---

1. Abbreviations: B(a)P, benzo(a)pyrene; HPLC, high performance liquid chromatography; TLC, thin layer chromatography.

0006-291X/78/0822-0518$01.00/0
Copyright © 1978 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Materials and Methods

Microsomal suspensions from the livers of 3-methylcholanthrene-treated rats were prepared as described previously (8). [^{14}C]-3-Hydroxy-B(a)P and [^{14}C]-B(a)P-9,10-dihydro-9,10-diol were isolated by HPLC techniques (9) from organic extracts of a microsomal incubation mixture containing [7,10-^{14}C]-benzo(a)pyrene; the radiolabelled 9,10-dihydrodiol was subsequently converted to 9-hydroxy-B(a)P using the method of Sims (10). 3- and 9-Hydroxy-B(a)P were obtained from the NCI Carcinogenesis Research Program.

A standard reaction mixture, containing 50 mM Tris-chloride buffer, pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 2 mM sodium isocitrate, 1 I. U. per ml of isocitrate dehydrogenase, 1 μM rotenone, 200 μM NADH and 0.5 mg/ml microsomal protein in a 20 ml volume, was preincubated at 37°. The 3- and 9-hydroxy-B(a)P were dissolved in acetone and added (<75 μl) to the reaction mixture (30 μM, final concentration) containing microsomal protein prior to initiation of the reaction with NADPH (400 μM, final concentration). Repetitive scan spectrophotometric analysis of the metabolic products was performed as described by Prough *et al.* (8). When radiolabelled 3- or 9-hydroxy-B(a)P (spec. radioactivity, approx. 3.2 mCi/mmole) was utilized, the experiments were performed identically to the procedure described for the metabolism of B(a)P (8,9) and analyzed using reverse phase HPLC followed by liquid scintillation counting.

Results

Spectrophotometric Measurement of the Metabolism of 3- and 9-Hydroxy-B(a)P As shown in Fig. 1a, the microsomal metabolism of 9-hydroxy-B(a)P is accompanied by a progressive loss of absorbance at approximately 421 nm concomitant with increases in absorbance at 409 and 436 nm; the negative absorbance change is due in part to the loss of 9-phenol. The two major 9-phenol metabolites described below have absorbance bands at 427 and 435 nm which could account for the absorbance increases above 425 nm during metabolism (Table 1). Appreciable absorbance is generated above 450 nm suggesting that a small amount of B(a)P-quinones may exist during the steady-state of the reaction. The time course of the reaction was linear for 6-8 min and an apparent K_m of approximately 15 μM was obtained from a Lineweaver-Burk plot. The presence of isosbestic points at 402, 416, and 426 nm suggests that the spectral changes noted during the metabolism of 9-hydroxy-B(a)P may be limited to one predominant product. The appearance of these spectral changes was dependent upon the presence of NADPH, microsomal protein, and oxygen indicating the involvement of an oxygenase reaction, possibly cytochrome P-450-dependent.

Experiments using 3-hydroxy-B(a)P yielded difference spectra which were

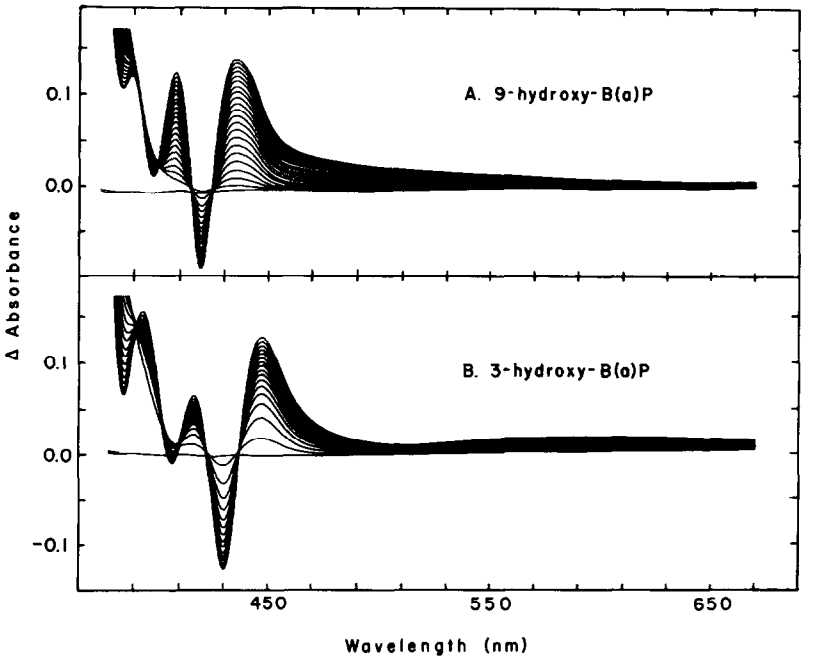

Figure 1. Difference Spectral Analysis of the Metabolism of the 3- and 9-Phenols of Benzo(a)pyrene. A reaction mixture containing B(a)P-phenols and rat liver microsomes from 3-methylcholanthrene-treated rats (see Methods) was bubbled with oxygen and divided into the sample and reference cuvettes of an Aminco DW-2 spectrophotometer thermostated at 37°. After a baseline of equal light absorbance was recorded in the split beam mode, NADPH (400 μM, final concentration) was added to the sample cuvette, and the time-dependent changes in absorbance between 370 and 470 nm were followed by repetitive scanning at 10 nm/sec. A. Metabolism of 9-hydroxy-B(a)P. B. Metabolism of 3-hydroxy-B(a)P.

similar to those obtained with the 9-phenol (Fig. 1b). Major absorbance maxima at 396, 420, and 449 nm and absorbance minima at 408 and 432 nm were developed during the aerobic steady-state of the reaction; the latter corresponds in part to the absorbance maxima of 3-hydroxy-B(a)P suggesting the loss of the phenol during the reaction. The spectrum was distinct from that of B(a)P-3,6-quinone in the presence of denatured microsomal suspensions, but was nearly identical to that of the sodium dithionite-reduced quinone (11) minus the spectrum of the amount of 3-phenol lost during metabolism (Fig. 2).

Isolation of Products Reaction mixtures containing radiolabelled

TABLE 1. PROPERTIES OF B(a)P METABOLITES AND STANDARDS

COMPOUND	RETENTION[1] TIME (min)	ABSORBANCE[2] MAXIMUM (nm)	EMISSION[3] MAXIMUM (nm)
3-hydroxy	1.00	424	431(520)
9-hydroxy	0.96	418	422(513)
3,6-quinone	0.83	480	442,570(570)
7,8-dihydrodiol	0.57	366	397(397)
4,5-dihydrodiol	0.48	322	375(375)
9,10-dihydrodiol	0.26	344	404(404)
Peak A (9-hydroxy)	0.59	435	442(533)
Peak B (9-hydroxy)	0.68	427	441(532)
Peak C (3-hydroxy)	0.59	435	442(533)
Peak D (3-hydroxy)	0.84	480	442,570(570)

1. The retention times are expressed relative to 3-hydroxy-B(a)P.
2. The absorbance wavelength noted is the longest wavelength maximum of the compounds (in methanol).
3. The fluorescence wavelength noted is the major emission wavelength maximum of the B(a)P derivatives in 80% methanol and the values in parentheses are the maxima in the presence of 0.15% triethylamine in 80% methanol. The samples were excited at 270 nm.

9-hydroxy-B(a)P were incubated and aliquots removed at various times. After extraction and concentration, the samples were analyzed using reverse phase HPLC and fractions were collected for further characterization. Figure 3 provides evidence for the formation of two major polar metabolites whose retention times relative to the 3-phenol are 0.59 (peak A) and 0.68 (peak B), respectively; these compounds have chromatographic properties similar to the 7,8-dihydrodiol of B(a)P (Table 1). The optical and fluorescence emission spectra of peaks A and B were similar to the spectra of 9-hydroxy-B(a)P (Table 1). In addition, the effect of base titration on the fluorescence emission spectra of peaks A and B was very similar to the effect seen with the 9-phenol. The presence of at least one very polar minor metabolite (>5%) was detected in the early portion of the gradient elution.

The metabolism of [^{14}C]-3-hydroxy-B(a)P was studied using similar HPLC techniques and two easily distinquished metabolites were observed. The major metabolite obtained by HPLC (peak D) had a retention time and an absorbance spectrum identical to that of B(a)P-3,6-quinone (Table 1), in agreement with

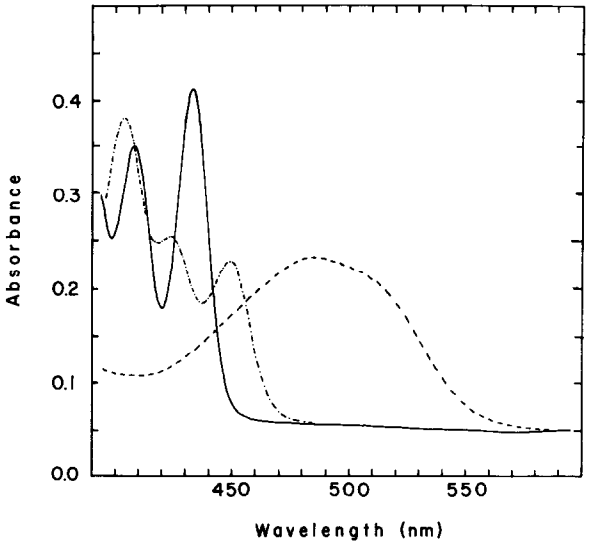

Figure 2. The Spectra of the Possible Metabolites of 3-Hydroxy-B(a)P During the Steady-State. The spectrum of B(a)P-3,6-quinone (13 μM) is shown in the presence of 1.5% Triton X-100, 0.15 M KCl, 0.05 M Tris chloride, and 5 mM $MgCl_2$, pH 7.5, before (– – –) and after (-·-·-) the addition of a few grains of sodium dithionite. The spectrum of 3-hydroxy-B(a)P (34 μM) in the presence of 1.5% Triton X-100, 0.15 M KCl, 0.05 M Tris chloride, and 5 mM $MgCl_2$, pH 7.5, is included for comparison (——).

the report of Wiebel (4). The second metabolite of moderate polarity had a retention time of 0.59 relative to 3-hydroxy-B(a)P (peak C) and had an absorbance spectrum identical to the peak A metabolite of 9-hydroxy-B(a)P (Table 1).

Discussion

Prough et al. (12) have utilized the method of repetitive scan difference spectrophotometry to monitor absorbance changes occurring during the oxidative metabolism of B(a)P. The method has utility in directly observing the formation of metabolic products during the steady-state of the reaction; this condition cannot be met by other analytical methods available. The analysis of metabolism using difference spectral and HPLC techniques indicates that the major products of 9-hydroxy-B(a)P appear to be compounds which most likely are diphenols that do not rapidly oxidize to yield quinones.

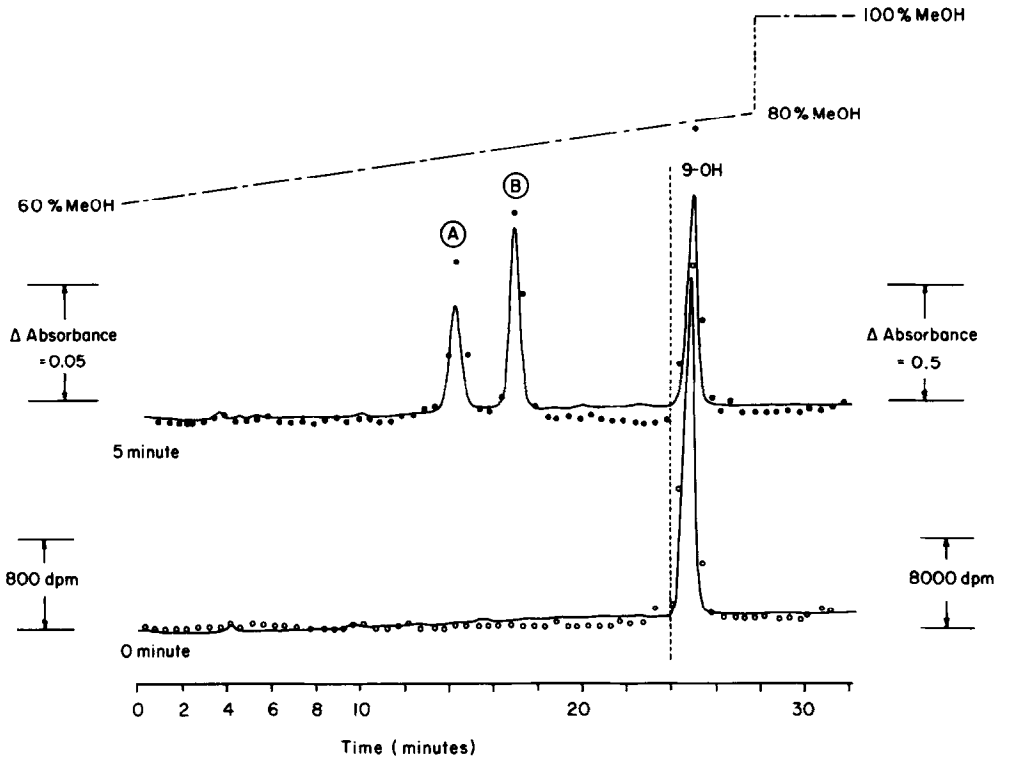

Figure 3. HPLC Separation of 9-Hydroxy-B(a)P and Its Metabolic Products. The dry residue of ethyl acetate extracts of the 15 minute reaction mixture (see Methods) containing 9-hydroxy-B(a)P was dissolved in methanol and analyzed using a reverse phase HPLC column (μBondapak C_{18}, Waters Associates) and a 60 to 80% methanol:water linear gradient (0.67% change/min;) at a 1.5 ml/min flowrate. The separation was monitored by 254 nm absorbance ( ) and samples were collected every 30 sec for radioactivity analysis (o o o, zero min reaction mixture or o o o, five min reaction mixture).

This interpretation is based on the fact that when the B(a)P nucleus is oxidized to yield an arene oxide or dihydrodiol, there is no significant absorbance or fluorescence emission spectra above 395 nm (12,13). The known B(a)P quinones have additional absorbance maxima above 450 nm and have fluorescence emission spectra which are distinct from those of the 1-, 3-, 6-, or 9-phenols (11,13). Although the major product of 3-hydroxy-B(a)P metabolism determined by HPLC is the 3,6-quinone, the product measured spectrophotometrically during the steady-state of the reaction most probably is B(a)P-3,6-dihydroquinone (diphenol); this product can react chemically

with oxygen to form the quinone and hydrogen peroxide (11). A second metabolic product (peak C, Table 1) exists which most likely is B(a)P-3,9-diphenol based on HPLC retention time, absorbance, and fluorescent emission spectra which are apparently identical to the product of 9-hydroxy-B(a)P metabolism (peak A). These results indicate that there exists within the liver microsomal fraction an oxygenase capable of oxidatively metabolizing B(a)P-phenols to diphenols. The formation of diphenols most likely would occur via an unstable phenol-epoxide intermediate which could have the capacity to form covalent bonds with cellular nucleophiles; this conclusion is consistent with the results of Capdevila *et al.* (6) and King *et al.* (7).

Our results support the contention that the relatively high amount of quinones obtained after HPLC analysis of the *in vitro* microsomal metabolism of B(a)P or 3-hydroxy-B(a)P may not represent the actual metabolic profile *in vivo* or during the steady-state of the *in vitro* reaction. Quinones are not major products of the metabolism of B(a)P by cells or intact animals ; large amounts of glucuronide conjugates are formed at the expense of phenols and quinones (14,15). The conversion of the 3,6-B(a)P-diol to the 3,6-dione may be the consequence of the manipulation and time involved in the analysis of samples by HPLC or TLC. The discrepancy between the *in vitro* microsomal metabolism and metabolism of B(a)P or the 3-phenol by intact cells or whole animals could be due in part to the formation of B(a)P-diols which are capable of being rapidly conjugated and excreted. The formation of additional metabolites during the oxidation of phenols of polycyclic hydrocarbons expands the complexity and range of compounds which may contribute to the carcinogenic and/or toxic potential of B(a)P.

Acknowledgements

This study was supported in part by USPHS Grant HL19654 (RAP) and NCI Contract NO1 CP 33362. RAP is the recipient of a USPHS Research Career Development Award, HL00255. The authors wish to express their appreciation to Mr. Richard Hillman and Ms. V.G. Patrizi for their technical assistance.

References

1. Nagata, C., Tagashira, Y., and Kodama, M. (1974) Chemical Carcinogenesis, Part A. (P.O.P. Ts'o and J.A. DiPaulo, eds.), pp. 87-111, Marcel Dekker, New York.
2. Lorentzen, R.J., Caspary, W.J., Lesko, W.J., and Ts'o, P.O.P. (1975) Biochem., 14, 3970-3977.
3. Lesko, S., Caspary, W., Lorentzen, R., and Ts'o, P.O.P. (1975) Biochem., 14, 3878-3983.
4. Wiebel, F.J. (1975) Arch. Biochem. Biophys., 168, 609-621.
5. Yang, S.K., Roller, P.P., Fu, P.P., Harvey, R.G., and Gelboin, H.V. (1977) Biochem. Biophys. Res. Communs., 77, 1176-1182.
6. Capdevila, J., Jernstrom, B., Vadi, H., and Orrenius, S. (1975) Biochem. Biophys. Res. Communs., 65, 894-900.
7. King, H.W.S., Thompson, M.H., and Brookes, P. (1976) Int. J. Cancer, 18, 339-344.
8. Prough, R.A., Patrizi, V.W., and Estabrook, R.W. (1976) Cancer Res., 36, 4439-4443.
9. Selkirk, J.K., Croy, R.G., and Gelboin, H.V. (1975) Arch. Biochem. Biophys., 168, 322-328.
10. Sims, P. (1968) J. Chem. Soc., C, 32-34.
11. Lorentzen, R.J. and Ts'o, P.O.P. (1977) Biochem., 16, 1467-1473.
12. Jerina, D.M., Yagi, H., Hernandez, O., Dansette, P.M., Wood, A.W., Levin, W., Chang, R.L., Wislocki, P.G., and Conney, A.H. (1976) Carcinogenesis, Vol. 1 (R.I. Freudenthal and P.W. Jones, eds.) pp. 91-113, Raven Press, New York.
13. McCaustland, D.J., Fischer, D.L., Kolwyck, K.C., Duncan, W.P., Wiley, J.C., Jr., Menon, C.S., Engel, J.K., Selkirk, J.K., and Roller, P.P. (1976) Carcinogenesis, Vol. 1 (R.I. Freudenthal and P.W. Jones, eds.) pp 349-411, Raven Press, New York.
14. Falk, H.L., Kotin, P., Lee, S.S., and Nathan, A. (1962) J. Nat. Cancer Inst., 28, 699-724.
15. Burke, M.D., Vadi, H., Jernstrom, B., and Orrenius, S. (1977) J. Biol. Chem., 252, 6424-6431.