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PRELIMINARY COMMUNICATIONS

ENZYMATIC CONVERSION OF BENZO(A)PYRENE PHENOLS, DIHYDRODIOLS AND QUINONES TO SULFATE CONJUGATES

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Benzo(a)pyrene(BP) is a ubiquitous carcinogen in our environment and is found in air, water and in the food chain, as well as in cigarette smoke condensate [1]. The biological activities of BP depend on its metabolism to active intermediates by the microsomal mixed-function oxidases and an epoxide hydratase [2-7]. Recently, an ultimate carcinogenic form of BP was reported to be r-7,t-8-dihydroxy-t-9,10-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene (diol-epoxide I) [8]. In tissue culture cells and *in vivo*, almost all the BP metabolites are converted to very polar forms which are not extractable with organic solvents [9-12]. Some of these metabolites have been thought to be conjugates of glutathione or glucuronic acid. The existence and some properties of the enzymes mediating the latter conjugation reactions were shown in our previous papers [13-15]. Another possible conjugation is with sulfate, via an activated form, 3-phosphoadenosine-5-phosphosulfate (PAPS). The sulfate conjugate of 3-hydroxybenzo(a)pyrene (3-OH BP) was found in the ethylacetate extracts of BP metabolites from human, rat and hamster lung cultures by Cohen *et al.* [16] and the enzyme system was further characterized in a subsequent study [17]. This paper reports the enzymatic conjugation with sulfate of a variety of oxygenated BP metabolites.

The sulfate conjugates were detected by methods described previously [17]. As the enzyme source, a 105,000 g supernatant solution was prepared from the livers of male Wistar rats. The reaction mixture, in a total volume of 0.1 ml, contained 40 mM Tris-HCl, pH 7.5, containing 100 μg of 105,000 g supernatant protein, 5 mM $MgCl_2$, 5 mM ATP

neutralized with NaOH, 2 mM ^{35}S-Na_2SO_4 (Amersham/Searle) and 1.25×10^{-4} M BP derivatives. The phenols were dissolved in methanol and other metabolites in tetrahydrofuran. *,+

The reaction mixture was incubated at 37^0 for 30 min without BP derivatives. This preincubation was performed so PAPS could be formed prior to the addition of the BP metabolites. The 30-min period was enough to get a constant reaction rate for sulfate conjugation. After addition of the derivatives, the reaction mixture was incubated for 10 min. The enzyme reaction was stopped with 2 vol. ethanol, and portions of aliquots were applied to Silica gel thin-layer sheets (J. T. Baker Co.).

On thin-layer chromatography (T.L.C.) using a solvent mixture of ethylacetate: methanol:water:formic acid, 100:25:20:1 by vol., unreacted BP derivatives were seen as spots at the solvent front and Na_2SO_4 was at a R_f of less than 0.1. Almost all of the ^{35}S-radioactivity bound to BP derivatives appeared at R_f = 0.6-0.7; it was difficult to separate one radioactive substance clearly from another except for the quinones. When BP quinones were used as substrates, the spots were located at R_f = 0.35. Although the presence of the conjugates was recognized by their fluorescence under U.V. illumination, the amounts were calculated by the ^{35}S-specific activities.

Table 1 summarizes two experiments on sulfate conjugation obtained with the various BP metabolites and derivatives. All of the phenols tested formed sulfate conjugates. The 10-OH BP and 2-OH BP showed the lowest amount of conjugate formation. The latter phenols have not been demonstrated as BP metabolites. The known metabolites 1-OH, 3-OH, 6-OH, 7-OH and 9-OH formed intermediate levels of sulfate conjugates.

The quinone metabolites also were found to form conjugates with sulfate. The BP-3,6-quinone showed two spots on the T.L.C. system; the major one was at R_f = 0.35 and the other minor spot at R_f = 0.65. The latter can be the conjugate of 3-OH or 6-OH BP with sulfate after a reduction from 3,6-quinone in the reaction mixtures. The sulfate conjugates of the phenols and diols were ethylacetate extracted, whereas the conjugates of the quinones were not extractable.

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* The amounts of conjugate formation with 3-OH BP or BP-7,8-oxide were identical whether they were dissolved in methanol or tetrahydrofuran.

+ Quinone derivatives were not completely dissolved in tetrahydrofuran at 2.5 mM, so that 5 µl was pipetted from saturated solutions.

Table 1. Formation of sulfate conjugates with benzo(a)pyrene metabolites and derivatives*

BP derivative	Amount of conjugate formed (nmoles/mg protein/10 min)	
	Expt. I†	Expt. II†
1-OH	5.90	3.65
2-OH‡	2.32	3.53
3-OH	4.93	6.87
4-OH	5.19	3.75
6-OH	9.23	7.87
7-OH	4.22	5.03
8-OH‡	7.17	6.03
9-OH	5.80	3.50
10-OH‡	0.67	0.86
12-OH‡	11.29	5.93
1,6-Quinone	5.35	2.92
3,6-Quinone	4.75	3.17
4,5-Oxide	0.19	0.50
7,8-Oxide	16.18	11.24
4,5-Dihydrodiol	3.03	1.96
7,8-Dihydrodiol	0.64	0.03
9,10-Dihydrodiol	1.10	0.39
Diol-epoxide I	Trace§	Trace§
Diol-epoxide II	Trace§	Trace§

*Incubation conditions were described in the text.

‡Not identified in literature as metabolite.

†The 105,000 g supernatant fractions were used from different animals. The mean and standard deviation of the enzyme activity from six animals using 3-OH BP were 5.12 and 1.43 respectively.

§Trace means only trace and not quantifiable amounts were detected. The abbreviations used here and in the text were as follows: 1-OH = 1-hydroxy-benzo(a)pyrene and numbers refer to positions on the benzo(a)pyrene ring; quinones, oxides and dihydrodiols are all derivatives of BP; diol-epoxide I is r-7,t-8-dihydroxy-t-9,10-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene and diol-epoxide II is r-7,t-8-dihydroxy-c-9,10-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene.

In rat liver perfusion experiments, Vadi et al. [12] observed BP metabolites in the perfusate and found that more than half of the metabolites were water-soluble. A large proportion of the metabolites in the ethylacetate extractable fraction were dihydrodiols. Although they also detected negligible amounts of BP phenols, they did not report BP quinones in the perfusate. Quinone metabolites have been reported formed with microsomal mixed-function oxidases. Our study indicates that these quinones may be excreted as sulfate conjugates. We do not know the mechanism of quinone conjugation to sulfate. Perhaps the quinones undergo a prior reduction. Thus, sulfate conjugation may be a key route of quinone disposition.

Three dihydrodiol metabolites formed sulfate conjugates, among which the 7,8-dihydrodiol sulfate conjugate was formed relatively slowly. Although the 7,8-oxide showed the highest amount of sulfate conjugation among BP derivatives examined in the present experiments, the K-region oxide, 4,5-oxide, was a poor substrate. BP-7,8-oxide is chemically very labile and is easily converted to 7-OH BP in aqueous solution. Therefore, BP-7,8-oxide might be forming the sulfate conjugate after being converted to the 7 phenol.

The reaction of diol-epoxide I or its stereoisomer diol-epoxide II occurred to a very small extent and only trace amounts were observed. For glutathione conjugation, diol-epoxide II reacted far more rapidly than diol-epoxide I (N. Nemoto and H. V. Gelboin, manuscript in preparation). An ultimate form of BP diol-epoxide I is derived from trans-7,8-dihydrodiol by epoxidation by the microsomal mixed-function oxidases. The diol-epoxide and the trans-7,8-dihydrodiol were both poor substrates for the sulfate conjugation in our present experiment and for glucuronyl transferase in a previous paper[15]. The amount of 7,8-dihydrodiol produced from BP with microsomes was not generally less than those of 4,5- and 9,10-dihydrodiols, but, because of its poor substrate capacity for the sulfate conjugation, this route of excretion for the 7,8-dihydrodiol might be slower than that of the other metabolites. The carcinogenic or mutagenic activity of BP might be the result of the balance between the activation and detoxification processes, and sulfate conjugation may play a significant role in tissue, specie and individual susceptibility to the carcinogenic action of polycyclic aromatic hydrocarbons.

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REFERENCES

1. Committee on Biologic Effects of Atmospheric Pollutants, Particulate Polycyclic Organic Matter, National Academy of Sciences, Washington, D.C. (1972).

2. A. H. Conney, E. C. Miller and J. A. Miller, J. biol. Chem. 228, 753 (1957).

3. P. Sims, Biochem. Pharmac. 16, 613 (1968).

4. N. Kinoshita, B. Shears and H. V. Gelboin, Cancer Res. 33, 1937 (1973).

5. J. K. Selkirk, R. G. Croy, P. P. Roller and H. V. Gelboin, Cancer Res. 34, 3474 (1974).

6. I. V. Wang, J. F. Rasmussen and T. T. Crocker, Biochem. biophys. Res. Commun. 49, 1142 (1972).

7. E. Boyland and K. Williams, Biochem. J. 94, 190 (1965).

8. E. Huberman, L. Sachs, S. K. Yang and H. V. Gelboin, Proc. natn. Acad. Sci. U.S.A. 73, 607 (1976).

9. L. N. Andrianov, G. A. Belitsky, O. J. Ivanova, A. Y. Khesina, S. S. Khitrova, L. M. Shabad and J. M. Vasiliev, Br. J. Cancer 21, 566 (1967).

10. L. Diamond, C. Sardet and G. H. Rothblat, Int. J. Cancer 3, 838 (1968).

11. K. L. Falk, P. Kotin, S. S. Lee and A. Nathan, J. natn. Cancer Inst. 28, 699 (1962).

12. H. Vadi, P. Moldeus, J. Capdevila and S. Orrenius, Cancer Res. 35, 2083 (1975).

13. N. Nemoto and H. V. Gelboin, Archs Biochem. Biophys. 170, 739 (1975).

14. N. Nemoto, H. V. Gelboin, W. H. Habig, J. N. Ketley and W. B. Jakoby, Nature, Lond. 255, 512 (1975).

15. N. Nemoto and H. V. Gelboin, Biochem. Pharma. 25, 1221 (1976).

16. G. M. Cohen, S. M. Haws, B. P. Moore and J. W. Bridges, Biochem. Pharma. 25, 2561 (1976).

17. N. Nemoto and S. Takayama, Biochem. Pharma., in press.