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ENZYMATIC FORMATION AND PROPERTIES OF A CONJUGATE OF SULFATE WITH 3-HYDROXYBENZO(A)PYRENE

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The powerful and ubiquitous carcinogen benzo(a)pyrene(BP) is metabolized to phenols, quinones, dihydrodiols, epoxides, and dihydrodiol-epoxide by NADPH-dependent mixed-function oxidases and epoxide hydratase in the microsomes (1-8). In addition, water-soluble metabolites of BP have been detected, especially in experiments in vivo (9, 10); less than half the metabolites of BP formed when BP was administered to tissue culture cells or animals could be extracted with organic solvents (11-14). The water-soluble metabolites were partially sensitive to β-glucuronidase and sulfatase, so it has been suggested that they include conjugates with glucuronic acid and sulfate (14, 15). Recently, a conjugate with sulfate was identified in the medium after incubating human tissue culture cells with BP (16). Conjugates of glutathione (GSH) with BP-epoxides may be formed also, because Waterfall and Sims (17) have reported that GSH reacts with these epoxides. A quantitative method for assay of these conjugates was developed very recently by Hayakawa and Udenfriend (18) and by us (19). We established also a method for assay of UDP-glucuronyl transferase using 3-hydroxybenzo(a)pyrene (3-OH BP), a major metabolite formed by microsomal mixed-function oxidases, as standard substrate (20). This paper is about the enzymatic formation of a conjugate of sulfate with 3-OH BP by rat liver 105,000 g supernatant.

MATERIALS AND METHODS

Tritium-labeled and cold 3-OH BP were generously given by Dr. H. V. Gelboin, N.I.H., USA. Rat liver 105,000 g supernatant was prepared from male Wistar rats, weighing about 150 g, as described previously (19). The reaction mixture in 0.1 ml of 40 mM Tris-HCl, pH 7.5, contained 5 mM ATP, 5 mM $MgCl_2$, 2 mM ^{35}S-Na_2SO_4 (5.6 mCi/mmole, The Radiochemical Centre, Amersham, England), 100 μg of 105,000 g supernatant and 1.25 X 10^{-4} M 3-OH BP in 5 μl of methanol. The mixture was preincubated at 37°C for 30 min without 3-OH BP; 10 min after addition of 3-OH BP, 2 vol. of cold ethanol was added to stop the reaction and the mixture was centrifuged to precipitate the proteins. Half the supernatant was applied to

a silica gel thin-layer chromatography sheet (J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A.) and the sheet was developed with a mixture of ethyl acetate : methanol : water : formic acid, 100 : 25 : 20 : 1 by vol. Unreacted 3-OH BP and Na_2SO_4 were found at the solvent front and at Rf 0.1 respectively. During the incubation, material formed which gave a spot with blue fluorescence at Rf 0.75. The radioactivity in this spot was counted as described previously (19).

RESULTS AND DISCUSSION

Of the enzymes tested only sulfatase digested the material in the new spot (Table 1). Commercial sulfatase has low β-glucuronidase activity but this did not affect the result, because the 105,000 g supernatant did not form a conjugate of glucuronide with 3-OH BP (20). When ^{3}H-labeled 3-OH BP was used, the spot had ^{3}H-radioactivity; similar values for the

Table 1. Effects of various enzymes on the conjugate

Enzyme	Radioactivity		
	Total cpm	In conjugate cpm	%
Bovine Serum Albumin*1	1743	1558	89
Acid Phosphatase*2	1690	1574	93
Pancreatic RNase*3	2893	2714	94
Sulfatase*4	3184	74	2

The fluorescence spot obtained as described in the text was extracted with ethanol : water (2 : 1, v/v) and then treated with various enzymes in 0.15 M sodium acetate buffer, pH 5.0 for 2 hr. Then the proteins were precipitated with ethanol, and the soluble fractions were subjected to TLC using the same system as before.

*1. Protein concentration, 3 mg/ml.

*2. Phosphatase (Type I, Sigma) concentration, 1.5 mg/ml.

*3. Enzyme (Sigma) concentration, 150 μg/ml.

*4. Sulfatase (Type V, Sigma) concentration, 300 μg/ml.

amount of conjugate formed were calculated on the basis of specific activity using ^{3}H-3-OH BP and ^{35}S-Na_2SO_4 (Table 2). These results show that the spot was probably that of a conjugate of sulfate with 3-OH BP. The fluorescence spectra of the material in the spot were slightly different from those of 3-OH BP. On excitation at 385 nm, peaks of fluorescence were observed

Table 2. Effects of incubation conditions on formation of the conjugate of sulfate with 3-OH BP

Reaction System		Conjugate (nmole/10 min-mg protein)
Exp. 1 Complete	a ^{35}S-Na_2SO_4	3.99
	b ^{3}H-3-OH BP	4.13
Exp. 2 Complete		4.13
-ATP		0
-$MgCl_2$		0.49
-Na_2SO_4		2.52
-105,000 g sup. + BSA		0
Exp. 3 Complete		5.76
EDTA 1 mM		3.00
EDTA 5 mM		2.07
EDTA 25 mM		1.40

Exp. 1. Incubations were carried out in the presence of either ^{35}S-Na_2SO_4 or ^{3}H-3-OH BP and the amount of conjugate was calculated from the specific activity of the substrate.

Exp. 2. Incubations were carried out using ^{3}H-3-OH BP.

Exp. 3. EDTA at the indicated concentration was added after preincubation.

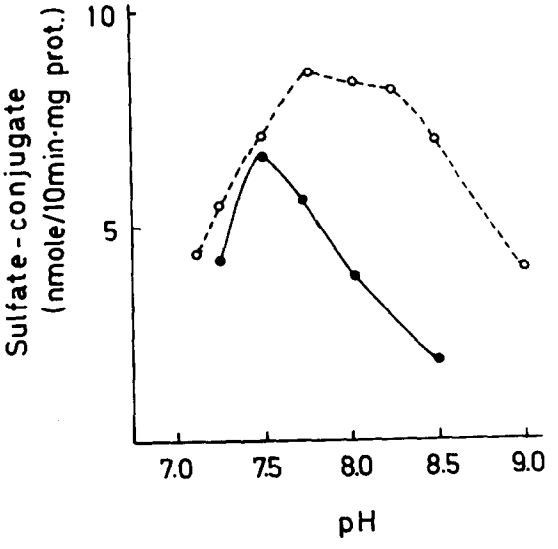
at 415 and 435 nm in aqueous ethanol; the fluorescence excitation spectra, with emission at 415 nm, had peaks at 268, 298, 370 and 385 nm. These fluorescence spectra were similar to those of synthetic benzo(a)pyren-3-yl hydrogen sulfate reported by Cohen et al (16).

The amount of sulfate conjugate formed depended on the amount of 105,000 g supernatant added and the incubation period. It also depended on the period of preincubation, but this did not need to be more than 20 min when the period of incubation with 3-OH BP was fixed at 10 min. ATP could not be replaced by AMP, GTP, or GMP, so the ultimate precursor for the sulfate conjugation must be 3'-phosphoadenosine 5'-phosphosulfate (PAPS), as indicated by Miller's group (21, 22). The enzyme reaction was dependent on the concentrations of $MgCl_2$ and ATP, possibly due to the formation of PAPS, but sulfate transferase itself may also be affected by the presence of $MgCl_2$, because addition of EDTA after preincubation decreased the amount of the conjugate (Table 2). The optimal pH for the sulfate-activating enzyme

was 7.5, but that for the sulfate transferase was pH 7.75. Figure 1 shows the effect of changing the pH after preincubation. After preincubation the amount of conjugate formed increased linearly for 10 min. The enzyme preparation was unstable on overnight dialysis, but was stable for at least 3 months on storage at -70°C.

The conjugate was less polar than other conjugates such as those of glutathione and glucuronide. About half of the conjugate could be extracted from aqueous solution with 2 vol. of ethyl acetate and all the rest could be recovered by further extractions with ethyl acetate. However, the conjugate could not be extracted from aqueous solution with benzene or n-hexane. It is of interest that Cohen et al. found a sulfate conjugate among the metabolites of BP extracted with ethyl acetate from the medium of human, hamster, and rat lung cells (16).

Fig. 1. Dependency of Conjugate Formation on pH



●—● pH not changed during incubation.

o--o pH fixed at 7.48 during preincubation and then changed to the indicated value by addition of HCl or NaOH.

When Na_2SO_4 was not added, the fluorescence spot which appeared at Rf 0.75 contained about half of the amount of conjugate, when ^{3}H-labeled 3-OH BP was used as substrate (Table 2). It has been reported that N-hydroxy-N-acetylaminofluorene (N-OH AAF) is able to bind nucleic acids by making an unstable intermediate phosphate ester (23). However, 3-

OH BP did not conjugate with phosphate from ATP, because the fluorescent spot was not affected by phosphatase and no radioactivity was incorporated into this material from γ-^{32}P-labeled ATP. The 105,000 g supernatant contains a small amount of endogenous SO_4^{--} ion. The exact amount of this was unknown. Because of this and the fact that the sulfate conjugate was formed in two steps it was impossible to calculate the Km value for Na_2SO_4. However, quite large amounts of conjugate were obtained at low concentrations of ^{35}S-Na_2SO_4.

The sulfate ester of N-OH AAF, which is considered as an ultimate carcinogen, is very unstable (21, 22), whereas, the sulfate conjugate of 3-OH BP reported here was rather stable. Thus the latter was easy to detect, whereas activity of sulfotransferase for N-OH AAF was scarcely detectable when the ester was trapped with nucleic acids or macromolecules. We think that the conjugation of 3-OH BP with sulfate may serve to detoxify the 3-OH BP. Moreover, as 3-OH BP is not known to be carcinogenic, the idea that formation of the sulfate ester of 3-OH BP is necessary for transport of 3-OH BP from the liver to target organs may be overlooked, whereas it should be tested.

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REFERENCES

1. A. H. Conney, E. C. Miller and J. A. Miller, J. Biol. Chem. 228, 753 (1957).
2. P. Sims, Biochem. Pharmacol. 16, 613 (1968).
3. N. Kinoshita, B. Shears and H. V. Gelboin, Cancer Res. 33, 1937 (1973).
4. J. K. Selkirk, R. G. Croy, P. P. Roller and H. V. Gelboin, Cancer Res. 34, 3474 (1974).
5. I. V. Wang, J. F. Rasmussen and T. T. Crocker, Biochem. Biophys. Res. Commun. 49, 1142 (1972).
6. E. Boyland and K. Williams, Biochem. J. 94, 190 (1965).
7. P. Sims & P. L. Grover, Adv. Cancer Res. 20, 165 (1974).
8. P. Sims, P. L. Grover, A. Swaisland, K. Pal and A. Hewer, Nature 252, 326 (1974).
9. F. Weigert and J. C. Mottram, Cancer Res. 6, 109 (1946).
10. H. L. Falk and P. Kotin, Clin. Pharmac. Ther. 4, 88 (1963).
11. L. N. Andrianov, G. A. Belitsky, O. J. Ivanova, A. Y. Khesina, S. S. Khitrovo, L. M. Shabad and J. M. Vasiliev, Bri. J. Cancer 21, 566 (1967).

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

13. M. Duncan, P. Brookes and A. Dipple, Int. J. Cancer 4, 813 (1969).

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

15. R. T. Williams, Detoxication Mechanisms, 2nd ed., Chapman and Hall, London (1959).

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

17. J. F. Waterfall and P. Sims, Biochem. J. 128, 265 (1972).

18. T. Hayakawa, R. A. Lemahieu and S. Udenfriend, Arch. Biochem. Biophys. 162, 223 (1974).

19. N. Nemoto and H. V. Gelboin, Arch. Biochem. Biophys. 170, 739 (1975).

20. N. Nemoto and H. V. Gelboin, Biochem. Pharmacol. 25, 1221 (1976).

21. J. R. DeBaun, J. Y. R. Smith, E. C. Miller and J. A. Miller, Science 167, 184 (1970).

22. J. R. DeBaun, E. C. Miller and J. A. Miller, Cancer Res. 30, 577 (1970).

23. J. A. Miller and E. C. Miller, Progr. Expt. Tumor Res. 11, 273 (1969).