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SEPARATION OF WATER-SOLUBLE METABOLITES OF BENZO[*a*]PYRENE FORMED BY CULTURED HUMAN COLON

HERMAN AUTRUP

Human Tissue Studies Section, Laboratory of Experimental Pathology, Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, MD 20014, U.S.A.

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Abstract—A method has been developed to separate conjugated metabolites of benzo[*a*]pyrene into three major fractions: sulfate esters, glucuronides and glutathione conjugates. In cultured human colon, formation of sulfate esters and glutathione conjugates is the major conjugation pathway, while formation of glucuronides accounts for only 6 per cent of the water-soluble metabolites. Hydrolysis of the sulfate esters with arylsulfatase and the glucuronides with β-glucuronidase released metabolites of benzo[*a*]pyrene that were extractable with organic solvent. Separation of these metabolites by high-pressure liquid chromatography indicated that *trans*-4,5-dihydro-4,5-dihydroxybenzo[*a*]pyrene,7,8,9,10-tetrahydro-7,8,9,10-tetrahydroxy benzo[*a*]pyrene and *trans*-9,10-dihydro-9,10-dihydroxybenzo[*a*]pyrene were the major substrates for UDP-glucuronic acid transferase, while *trans*-7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene and 9-hydroxybenzo[*a*]pyrene were the major substrates for sulfotransferase in cultured human colon.

The metabolism of benzo[*a*]pyrene (BP), an environmental procarcinogen, has been studied extensively in both microsomal preparations and in cells and various organs from several animal species [1–6]. The main emphasis of these studies has been to determine the pathways of activation into the ultimate carcinogenic form. However, the toxicity of a chemical to cells depends not only on the ability of the cell to activate the chemical but also on its ability to deactivate the chemical. One of the presumed deactivation pathways for BP is the conjugation of primary BP metabolites with polar groups, e.g. sulfate, glucuronic acid or glutathione which facilitates the excretion of chemicals, although the primary metabolites have also been shown to be excreted [7]. In a cell-free system, Nemoto *et al.* [8, 9] reported that most of the primary metabolites of BP can be conjugated with both sulfate and glucuronic acid. Cohen *et al.* [10, 11] have shown that the sulfate-conjugate of 3-OH BP* formed in cultured rat, hamster or human lung was extracted into ethylacetate. Treatment of the water-soluble metabolites remaining after extraction with organic solvent with β-glucuronidase converted these metabolites into organic-solvent extractable forms. Further characterization of these metabolites indicated that glucuronides of BP-phenols are the major BP conjugates in cultures of both rat hepatocytes and hamster embryo cells [12–14]. We now report a method for separation by liquid chromatography of the three major groups of conjugates of BP.

* Abbreviations: 3-OH BP, 3-hydroxybenzo[*a*]pyrene, 9-OH BP, 9-hydroxybenzo[*a*]pyrene; *trans*-4,5-diol, *trans*-4,5-dihydro-4,5-dihydroxybenzo[*a*]pyrene; *trans*-7,8-diol, *trans*-7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene; *trans*-9,10-diol, *trans*-9,10-dihydro-9,10-dihydroxybenzo[*a*]pyrene; (7,10/8,9)-tetrol, tetrahydroxytetrahydrobenzo[*a*]pyrene in which the 10-hydroxy is *cis* and 8-hydroxy and 9-hydroxy are *trans* to the reference 7-hydroxy, respectively (other tetrols are similarly designated).

MATERIALS AND METHODS

Radioactively labeled chemicals were obtained from Amersham/Searle, Arlington Heights, IL. β-Glucuronidase and arylsulfatase (from *Helix pomatia*) were obtained from Boehringer/Mannheim Biochemicals, Indianapolis, IN. Metabolites of BP were supplied by NCI Chemical Repository, Bethesda, MD. Media and antibiotics were supplied by the Grand Island Biological Co., Grand Island, NY.

Specimens and conditions of incubation. Non-tumorous human colonic tissues obtained at the time of either surgery or "immediate autopsy" [15] were immersed in L-15 tissue culture medium containing penicillin G (100 units/ml) and streptomycin (100 μg/ml) and kept at 4° for 3–20 hr until cultured. Explants (5 × 5 mm) of grossly normal appearing colon were cultured in a chemically defined medium as described previously [16]. The explants were cultured for 18–24 hr prior to the exposure to [^{3}H]BP (1.5 μM; 20 or 24 Ci/m-mole) for 24 hr. To identify the chromatographic behavior of the different types of conjugates, the explants were cultured in CMRL-1006 media without Na_2SO_4 and Na-glucuronate. For indentification of the glutathione-conjugates, explants were incubated in modified CMRL-1066 containing [^{35}S]L-cysteine (16 μCi/ml; 73 mCi/m-mole) for 18–24 hr prior to incubation with [^{3}H]BP. For the other types of conjugates [^{35}S]Na_2SO_4 (5 μCi/dish; 115 mCi/m-mole) or [^{14}C]glucuronic acid (1 μCi/ml; 100 mCi/m-mole) was added concomitantly with [^{3}H]BP.

Separation of the conjugates. A modification of the procedure of Metzler [17] for the separation of diethylstilbestrol conjugates was used. To 5 ml of the medium from incubation, 15 ml of ice-cold ethanol was added. The solution was kept at 4° for 30 min and then centrifuged at 9000 *g* for 15 min to remove the protein precipitate. The supernatant fraction was evaporated to dryness *in vacuo* at ambient temperature. The residue

was redissolved in 2 ml of 70% ethanol and applied to an alumina column (150 × 15 mm; Neutral; Brockmann Activity 1, 80–200 mesh). The column was eluted using a flow rate of 2 ml/min, first with 100 ml of absolute ethanol to remove BP and non-conjugated metabolites, then with 100 ml of water to elute BP-sulfate esters, followed by 100 ml of 0.05 M ammonium phosphate buffer (pH 3) to elute BP-glucuronides and finally with 25% formic acid to elute BP-glutathione and the more polar conjugates. Five-ml fractions were collected and the radioactivity was determined in 200-μl aliquots.

The fractions containing either the BP-sulfate esters or the BP-glucuronides were combined, and evaporated *in vacuo* using azeotropic distillation with ethanol. After redissolving the BP-sulfate esters and the BP-glucuronides in 20 ml of 0.1 M Na-acetate buffer (pH 4.6), the solutions were treated with arylsulfatase (1 unit/ml) and β-glucuronidase (2.5 units/ml), respectively, flushed with nitrogen for 5 min, and incubated in sealed tubes overnight at 37°. The solutions were then extracted three times with ethylacetate/acetone (2/1), dried over $MgSO_4$ and evaporated to dryness. The residue was dissolved in methanol and the metabolites were separated by Spectra Physics B-3500 high-pressure liquid chromatography (h.p.l.c.) using a Partisil 10 ODS-2 column (250 × 3.2 mm; Altex Scientific Inc., Berkeley, CA). The BP metabolites were eluted using a gradient of 60% methanol in water to 100% methanol with a rate change of 2%/min [18]. Then 0.3-ml fractions were collected and the radioactivity was determined. Optical markers of authentic BP metabolites were included as standards.

RESULTS

The separation of the BP metabolites using an alumina column and a 4-step gradient is shown in Fig. 1. The first peak contains BP and non-conjugated BP metabolites. The metabolite pattern of BP in this fraction is similar to that observed after direct extraction from the media with organic solvent (unpublished observations). Chromatography of the metabolites after co-incubation of the explants with both [^{3}H]BP and $^{35}SO_4$ showed that peak 2 contained the sulfate esters; $Na_2{}^{35}SO_4$, when chromatographed alone under similar conditions, was first eluted by the formic acid. Attempts to separate the different sulfate esters using either reverse-phase or reverse-phase paired-ion h.p.l.c. were unsuccessful. The glucuronide conjugates were eluted by ammonium phosphate buffer (pH 3), while 25% formic acid was required to elute the free glucuronic acid. Peak 4 contained the glutathione conjugates. Separation of this fraction by paired-ion chromatography revealed at least eight metabolites; these metabolites have different ratios of ^{3}H/^{35}S indicating several different types of conjugates. One metabolite did not contain any ^{35}S. The chemical natures of these metabolites are currently being investigated.

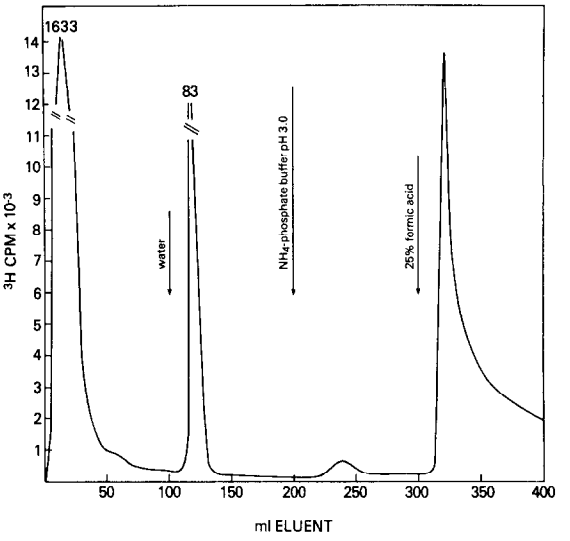

Fig. 1. Separation of BP metabolites formed by human colon using an alumina column (150 × 15 mm). The BP and non-conjugated metabolites were eluted with ethanol (100 ml), then the sulfate esters with water (100 ml), followed by the glucuronides which were eluted by ammonium phosphate buffer. Glutathione conjugates were eluted with 25% formic acid.

The results (Table 1) show that the water-soluble metabolites account for approximately 15 per cent of total BP metabolism in cultured human colon. Separation of the BP conjugates from eight different patients (Table 1) indicates that sulfate esters (45.3 ± 5.0 per cent of water-soluble metabolites; eight cases; mean ± S.D.) and glutathione conjugates (48.1 ± 7.8 per cent) are the major metabolic pathways in cultured human colon, while glucuronides only account for approximately 6 per cent of the total BP conjugates

Table 1. Separation of water-soluble metabolites into sulfate esters, glucuronides and glutathione conjugates

Patient	Total metabolism (pmoles)	Water-soluble metabolites (pmoles)	Sulfates	Glucuronides	Glutathione
C107	327	46	53.2*	2.0*	44.8*
C113	161	56	47.2	6.1	46.7
C123	267	28	40.2	2.7	57.1
C124	457	33	44.8	1.9	53.3
C125	75	36	47.1	15.3	37.6
C128	334	24	37.2	5.4	57.4
C131	225	63	44.3	13.8	41.3
C132	411	41	48.7	4.6	46.7

* Expressed as a percentage of total conjugated metabolites.

Table 2. Benzo[*a*]pyrene metabolites extractable into ethylacetate/acetone after treatment with either β-glucuronidase or arylsulfatase*

Metabolite	Retention time (min)	Glucuronide conjugates	Sulfate esters
(7,10/8,9)-Tetrol	3.6	14.8 ± 5.2†	6.2 ± 2.2
(7,9/8,10)-Tetrol	9.7	4.8 ± 4.1	7.4 ± 2.2
(7,9,10/8)-Tetrol + *trans*-9,10-diol	12.5	10.2 ± 6.7‡	5.1 ± 1.3
trans-4,5-diol	18.2	5.6 ± 5.5	1.4 ± 2.2
trans-7,8-diol	20.1	5.8 ± 2.5	12.1 ± 11.6
9-OH BP	25.2	8.2 ± 7.7§	17.9 ± 4.2
1-OH BP, 3-OH BP, 7-OH BP	26.2	5.8 ± 2.5	6.1 ± 1.8
Quinones	27.2–33.4	11.3 ± 5.8§	4.1 ± 1.8

* Percentage of total metabolites; mean ± S.D. of six experiments.
† Significantly different from sulfate esters; $P < 0.01$ (Student's *t*-test, two-tail).
‡ Significantly different when compared to sulfate esters; $P < 0.1$.
§ Significantly different when compared to sulfate esters; $P < 0.05$.

(6.5 ± 5.2 per cent). Treatment of the sulfate ester and the glucuronide fractions with hydrolytic enzymes released 68–94 and 57–60 per cent of the radioactivity, respectively, into ethylacetate–acetone (2:1) extractable metabolites.

Separation of the metabolites released by enzyme treatment by h.p.l.c. (Table 2) indicates the conjugating enzyme system has different specificities for the various BP metabolites. Significantly higher levels of (7,10/8,9)-tetrol, (7,9,10/8)-tetrol, 9,10-diol BP, and BP-quinones were conjugated with glucuronic acid, while higher levels of *trans*-7,8-diol and 9-OH BP were found in the form of sulfate ester. *Trans*-4,5-diol, (7,9/8,10)-tetrol, and 3-OH BP were conjugated by both systems.

DISCUSSION

BP is presumably inactivated in cells and tissues by conjugation of its metabolites with either glucuronic acid, sulfate or glutathione catalyzed by uridine diphosphate-glucuronyltransferase, sulfotransferase and glutathione transferase respectively. The liver is the primary site for these enzyme catalyzed reactions; however, other organs also contain these enzymes. Indirect methods using successive treatment of the media with hydrolytic enzymes have been used to determine the relative importance of the different pathways in the conjugation of BP metabolites. One of the advantages with the reported method is that no prior extraction of the media with organic solvents, which have been shown to extract some sulfate esters [10], is needed. Second, tritium, released from BP when phenols and quinones were formed, was removed by the azeotropic distillation, and finally, it gives a direct measurement of the three major groups of water-soluble metabolites.

In cultured human colon from different donors, approximately 10–50 per cent of the metabolites was found in the water layer after extraction with organic solvents, similar to an earlier observations using the extraction procedure [19]. By using a four-step liquid chromatography method, we found that sulfate esters and glutathione conjugates are the major water-soluble metabolites formed in cultured human colon, while BP-glucuronides accounted for about 6 per cent of the metabolites. By using the indirect methods, other researchers found that glucuronidation was the major conjugation pathway in hamster embryo cells (40 per cent of the conjugated metabolites; [14]) and in isolated rat hepatocytes (32 per cent [12, 13]). The relative level of BP-glucuronides formed by cultured human colon was similar to the relative level formed in perfused rat lung [20] and in cultured human bronchus (H. Autrup, unpublished results).

Glutathione conjugation is a major detoxification pathway in cultured human tissues, accounting for more than 40 per cent of the conjugates in human colon, and an even higher level in cultured bronchus (unpublished results); this pathway was responsible for less than 25 per cent of the total conjugates in isolated hepatocytes [12]. One possible explanation for this difference could be that more BP-epoxides, the major substrates for glutathione transferase [21], are formed in human tissues than are formed by isolated rodent liver. The level of epoxide hydrase in human tissues is higher than in the same tissue in rats [22, 23], but lower than in rodent liver [24]. We have shown previously that both diols and tetrols are the major metabolites of BP formed when human tissues are incubated with BP for 24 hr [19, 24]. Inter-individual variation in the relative distribution of conjugates was also seen, the amount of glucuronides ranging from 1.9 to 15.3 per cent (eight cases) of the total amount of conjugates. The intra-individual variation due to the methodology was less than 5 per cent (unpublished results), and the viability of the tissue, at the end of the experiment as monitored by high-resolution light microscopy, was good in all reported cases. A 100-fold inter-individual variation has been observed previously in the binding level of BP to DNA in human colon [16], and inter-individual variation in the relative amount of organic solvent-extractable metabolites was also observed [19].

Treatment of the isolated BP-glucuronides with β-glucuronidase released approximately 70 per cent of BP metabolites into organic solvent-extractable metabolites. This suggests that the glucuronide fraction contains minor conjugation products of a different origin or that tritium exchange between [^{3}H]BP and water took place during the 18-hr incubation with β-glucuronidase. Separation of these organic solvent-extractable metabolites by h.p.l.c. showed that BP tetrols or BP diol epoxide are the major substrates for UDP-glucuronyl-

transferase in cultured human colon. This observation is in contrast to findings in hamster embryonic cells and rat hepatocytes [12–14] where 3-OH and 9-OH BP were the major conjugates of glucuronic acid. BP-phenols and 7,8-epoxide of BP were found to be good substrates for UDP-glucuronyltransferase in cell-free systems, while the tetrols and the *trans*-7,8-diol were poor substrates [8]. When compared to glucuronides, significantly higher levels of both 3-OH BP and 9-OH BP were found conjugated with sulfate. This difference in metabolites between the sulfate esters and glucuronides suggests that a substrate specificity for the conjugating enzymes may exist. Substrate specificity for the conjugating systems *in vivo* has been shown previously [25]. By using the liquid chromatography method described in this paper, information on metabolism of BP into the three major groups of water-soluble metabolites can be obtained. Determination of these pathways in human tissues is important since conjugation of toxic metabolites is an integral part of the cellular defense against environmental carcinogens. However, it could also be used as a transfer mechanism to transfer the metabolites to a different cell type, where they could be reactivated into metabolites which bind to cellular macromolecules. It has been shown recently that the glucuronide of 3-hydroxy BP could be activated by a cell-free system into a DNA binding metabolite [26]. Glucuronides of carcinogens may be important in kidney and bladder carcinogenesis [27] and in colon carcinogenesis [28, 29].

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