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PRELIMINARY COMMUNICATIONS

ENZYMATIC CONJUGATION OF BENZO(a)PYRENE OXIDES, PHENOLS AND DIHYDRODIOLS WITH UDP-GLUCURONIC ACID

Nobuo Nemoto and Harry V. Gelboin
Chemistry Branch, National Cancer Institute, Bethesda, Md. 20014, U.S.A.

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Benzo(a)pyrene is a polycyclic aromatic hydrocarbon, which is a common environmental pollutant and carcinogen in experimental animals (1). Polycyclic aromatic hydrocarbons are metabolized to reactive carcinogenic and mutagenic intermediates as well as to detoxified products by the microsomal mixed-function oxidases and metabolically related enzymes. The pathways and extent of metabolite formation are thus intimately related to carcinogenic activity. The microsomal aryl hydrocarbon hydroxylase system, which includes the mixed-function oxygenases and epoxide hydratases, converts benzo(a)pyrene to epoxides, phenols, dihydrodiols, and quinones (2-7). In experiments with isolated microsomes, small amounts of water-soluble metabolites are also formed (8,9). With cells in culture and systems in vivo, the amount of water-soluble metabolites formed from BP is much higher relative to the organic solvent extractable metabolites (10-13). For example, bile and urinary metabolites of BP are largely in the form of water-soluble conjugates (14,15). Since the conjugation step may be of great importance to the removal of carcinogenic, mutagenic and toxic intermediates, we have sought to define the enzymatic basis of conjugate formation. The level of these enzymes may relate to an individual's ability to detoxify reactive intermediates of benzo(a)pyrene and hence to individual susceptibility to carcinogen action. In a previous report, we showed the presence of benzo(a)pyrene oxide S-glutathione transferase in the liver soluble fraction (16) and the transferase activity of seven pure homogeneous enzymes from rat liver and human liver in the conjugation of glutathione with benzo(a)pyrene 4,5-oxide (17). In this study, we report that a series of hydroxylated metabolites including phenols, dihydrodiols and epoxides of benzo(a)pyrene form glucuronide conjugates in the presence of UDP-glucuronic acid and microsomes.

Microsomal enzyme fractions were obtained from the livers of male Sprague-Dawley rats by ultracentrifugation of the postmitochondrial fraction at 105,000 g for 60 min. The standard incubation contained 5×10^{-5} M ^{3}H-labeled or non-radioactive 3-OH-BP (or other substrates), 1.5 mM non-radioactive or uridine diphosphate [^{14}C]-glucuronic acid (UDPG), 50 μg microsomal protein and 5 mM $MgCl_2$ in 0.2 ml of 20 mM Tris-HCl buffer, pH 7.5. The mixture was incubated for 30 min at 37° and the reaction was stopped by the addition of cold acetone; the amount of conjugate formed was calculated after its isolation by Silica gel thin-layer chromatography (Eastman Kodak) with a solvent mixture of ethyl acetate-methanol-water-formic acid, at a ratio of 100:25:20:1. With this chromatographic system, the conjugate product migrated with an Rf of 0.8. The unreacted 3-OH-BP or UDPG migrated with the solvent front and an Rf of 0.1-0.2 respectively. The product contained both ^{3}H and ^{14}C when the incubation was performed in the presence of [^{3}H]-3-OH-BP and ([^{14}C]-glucuronic acid) UDPG. The amount of glucuronide formed was identical when calculated on the basis of either the specific activity of ^{3}H or ^{14}C. The molar equivalent in the conjugate of 3-OH-BP and glucuronic acid was identical. Treatment with β-glucuronidase decreased the amount of the product (Table 1). The product was not extractable into ethyl acetate. These results suggest that the product formed during the incubation is a glucuronide conjugate of 3-OH-BP.

The reaction was linear with incubation time for 60 min and the amount of the conjugate formed was linear in the range of microsomal protein concentration from 5 to 50 μg protein. No conjugate was formed when either bovine serum albumin or the 105,000 g liver supernatant replaced the microsomes. Thus, conjugate formation is mediated by microsomal bound enzymes. Enzyme activity was affected by treatment with compounds which destroy or digest the microsomal matrix. We used two proteinases, trypsin and pronase (Table 1). Treatment with trypsin slightly enhanced the enzyme activity, and treatment with pronase destroyed most of the activity. Pronase digests peptide bonds more randomly than trypsin, and the milder digestion by trypsin may expose the enzyme to make it more available to substrate.

Table 1. Effects of various treatments on the conjugation of 3-hydroxybenzo(a)pyrene with UDPG

Incubation conditions		Formation of glucuronide conjugate (nmoles/mg protein)
Expt. 1	Control	33.7
	β-Glucuronidase (pre-treatment)	2.6
	β-Glucuronidase (post-treatment)	10.4
Expt. 2	Control	29.7
	Minus microsome + bovine serum albumin	0
	Minus UDPG	0.1
	Microsome pretreatment 100° for 5 min	0.1
Expt. 3	Control	30.8
	Trypsin (200 μg)	36.2
	Pronase (200 μg)	6.5
Expt. 4	Control	30.4
	Triton X-100 (0.025%)	16.1
	Triton X-100 (0.1%)	3.6
Expt. 5	Liver	30.5 ± 7.4
	Lung	7.3 ± 4.4
	Kidney	5.0 ± 1.8

Severe digestion by pronase may destroy the enzyme. After treatment with Triton X-100, the enzyme activity decreased drastically (Table 1). This may be due to inactivation of the enzyme or to a change in the ionic environment which prevents access of the benzo(a)pyrene derivative to the enzyme. Treatment with Triton X-100 (0.025%) has been reported to enhance the glucuronidation of p-nitrophenol (18). We observed a 9-fold stimulation of p-nitrophenol conjugation and a 50 per cent decrease in 3-OH-BP conjugation. Thus, the enzyme conjugating the benzo(a)pyrene derivative may be different than that conjugating p-nitrophenol. The character and relationship of this enzyme to the UDPG transferase system have not been established. It may well be related although not identical to the UDPG transferase described for other substrates (19-24). We also examined the relative activity of this enzyme system in liver, lung and kidney microsomes and found lung and kidney to exhibit activity at about 1/5 to 1/7 the level in liver.

Table 2 shows the conjugation of a number of known metabolites and synthetic oxygenated derivatives of benzo(a)pyrene. All of the phenols tested were conjugated to about the same extent except 8-OH, 2-OH, 4-OH and 6-OH which were conjugated to a lesser extent. 3-OH-BP is a major metabolite and 9-OH-BP is a metabolite

Table 2. Formation of glucuronide conjugates with various oxygenated metabolites and derivatives of benzo(a)pyrene*

BP derivatives	Amount of conjugates (nmoles/mg protein/30 min)
1-OH	29.6
2-OH	9.6
3-OH	32.0
4-OH	9.6
6-OH	12.0
7-OH	35.4
8-OH	7.0
9-OH	36.2
10-OH	35.8
12-OH	20.2
cis-4,5-diol	2.0
trans-4,5-diol	9.0
trans-7,8-diol	2.4
trans-9,10-diol	0
1,6-dione	0
3,6-dione	0
6,12-dione	0
4,5-oxide	6.4
7,8-oxide	26.8

*BP derivatives at a concentration of 5×10^{-5} M were incubated at 37° for 30 min with 50 g rat liver microsomes, 5 mM $MgCl_2$ and 1.5 mM ^{14}C -UDPG in 0.2 ml of 20 mM Tris-HCl, pH 7.5. The amounts of conjugates were determined as described in the text. All of the above derivatives were synthesized by published methods by Midwest Research Institute on NCI Contract No. 1-CP-33387. Small quantities of these compounds are available upon request to: Dr. Marcia Litwack, Mgr., Information and Resources Segment, National Cancer Institute, Bethesda, Md., 20014.

generally found in somewhat lesser amounts (4,6). Recently, studies with a new method of high-pressure liquid chromatography have isolated and identified 1-OH-BP and 7-OH-BP as benzo(a)pyrene metabolites (25). The other examined phenols have not been isolated as metabolites, although the metabolic formation of 6-OH-BP can be deduced from the known activation of the 6 position (26) and the isolation of 1,6-quinone, 3,6-quinone and 6,12-quinone. We found that each of the four metabolically formed

phenols are conjugated by the UDPG transferase system. No conjugation of quinone metabolites was detected. The 4,5-oxide ("K-region") is also conjugated. This conjugation, however, may be via the dihydrodiol which is formed by BP 4,5-oxide hydratase, another microsomal enzyme (27). The 7,8-oxide is conjugated to a large extent, greater than that observed for the corresponding dihydrodiol formed by the epoxide hydratase. The pathway of conjugation may be determined by the structural characterization of the conjugate.

The 7,8-diol has been shown to be the most active metabolite which binds to DNA in the presence of active microsomes (6). The synthetic 7,8-diol-9,10-oxide was found to be the BP derivative most active in the ability to bind to DNA (28). In experiments testing direct mutagenicity, we found that the 7,8-diol-9,10-oxide was about 3500- to 4000-fold more potent as a mutagen in mammalian cells than either benzo(a)pyrene or the K-region BP-4,5-oxide (29). The 7,8-diol-9,10-oxide has also been shown to be a metabolite and its precursor is the 7,8-diol (29). We also found that the 7,8-diol was the most active metabolite converted to a mammalian cell mutagen by metabolic activation (29). These studies suggest that a specific form (29) of the diol oxide may be the active carcinogenic form of benzo(a)pyrene. The latter was also most active as a bacterial mutagen when activated by microsomal enzymes (30), although in the latter report the 7,8-diol-9,10-oxide was no more active than the 4,5-oxide. The 7,8-dihydrodiol is conjugated to a lesser extent than the phenol metabolites and the K-region 4,5-diol. Thus, once formed, the 7,8-diol may be more susceptible to conversion to the potent mutagen (and carcinogen?) 7,8-diol-9,10-oxide than the glucuronide conjugate.

This report establishes directly the enzymatic basis for the glucuronidation of the oxide, phenol and dihydrodiol metabolites of benzo(a)pyrene. This pathway of polycyclic hydrocarbon metabolism may be of key importance as a determinant of polycyclic hydrocarbon carcinogenicity.

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