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BENZO(a)PYRENE QUINONE METABOLISM IN TRACHEAL ORGAN CULTURES

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SUMMARY: Tracheal organ cultures metabolized a mixture of benzo(a)pyrene quinones (BP-quinones) into products which were not extracted with acetone/ethyl acetate and were water-soluble. When the culture medium was extracted of the organic soluble metabolites and the water-soluble metabolites exposed to β-glucuronidase, BP-quinones and an unidentified highly polar metabolite was released. When the results were evaluated quantitatively it was found that 30% of the water-soluble metabolites were glucuronide conjugates. The remaining 70% of the aqueous soluble radioactivity was not sensitive to β-glucuronidase and was not identified. When BP was used as the substrate in tracheal organ culture, treatment of the aqueous soluble metabolites with β-glucuronidase resulted in the release of some BP-quinones. It has previously been assumed that BP-quinones are "end-products" of BP metabolism, however, this report clearly demonstrates that BP-quinones are subject to further metabolic processes.

Benzo(a)pyrene (BP) is converted by the mixed-function oxidase system of the endoplasmic reticulum into a variety of oxygenated derivatives. A primary enzymatic oxidation sequence utilizing NADPH and oxygen results in epoxide formation and subsequently these are converted to dihyrodiols through enzymatic epoxide hydration (1). Monohydroxy benzopyrenes (BP-phenols) have been postulated to be generated via spontaneous rearrangement of the epoxide although the hypothesis that they are directly hydroxylated is still under consideration (2). The occurrence of BP-quinones and the role of the mixed-function oxidase complex in their formation is not well understood. Lesko et al., (3) and Lorentzen et al., (4) have observed the formation of 6-OH BP and the auto-oxidation of this compound to 6-oxobenzo(a)pyrene radical and subsequent oxidation to BP-quinones. Capdevila et al., (5) suggest that

ABBREVIATIONS: BP, benzo(a)pyrene; HPLC, high pressure liquid chromatography.

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BP-quinones result from sequential enzymatic oxygenation of BP to mono and to dihydroxy-BP, which auto-oxidizes to BP-quinones.

BP-quinones comprise a major class of metabolites produced in some isolated microsome systems in vitro (6-10). We have previously reported (11) that approximately 50% of the ethyl acetate extracted metabolites produced by tracheal epithelial microsomes co-chromatograph with 1,6-, 3,6-, and 6,12-BP quinones. However, tracheas incubated with [^{3}H]BP do not appear to produce BP-quinones in significant quantity in comparison with other metabolites. The possibility exists that BP-quinones are indeed initially produced by microsomal oxidation in trachea in vivo but that quinones are metabolized to non-ethyl acetate soluble products such as conjugates or recycled to water-soluble polyhydroxylated derivatives. This may explain, at least in part, the paucity of BP-quinones recovered following metabolism in organ cultures. In this report we tested the hypothesis that BP-quinones can be further metabolized by tracheal organ cultures and show that they can be recovered from the aqueous phase after treatment of the organ culture media with β-glucuronidase.

Materials and Methods

Syrian golden hamsters of about 100 g. were obtained from ARS Sprague-Dawley (Madison, WI). Tracheas were removed by blunt dissection from animals which had been anesthetized with ether. Excised tracheas were freed of surrounding connective tissue, severed through the trachealis muscle, and then placed in Ham's F-10 media (GIBCO, Grand Island, NY) buffered to pH 7.4 with HEPES and sodium bicarbonate and maintained at 37° in an atmosphere of 5% CO_2/95% air.

A mixture of [^{3}H]BP-quinones was produced by two methods. [^{3}H]BP (27 Ci/mmole; Amersham, Arlington Heights, IL) was applied to a thin-layer chromatogram sheet of silica gel G (Eastman, Rochester, NY), exposed to a germicidal UV lamp for 5 min and allowed to remain in air for three days. After this time, the yellow spot consisting of BP had turned greenish-brown in visible light. Oxidized derivatives of BP remained at the origin and were separated from BP by chromatographing in benzene/hexane 1:1. BP-quinones were also isolated as impurities present in [^{3}H]BP as obtained from the radiochemical manufacturer by applying the [^{3}H]BP to a silica gel column and eluting with hexane. The yellow-orange BP-quinones and other oxidized products remained in a discrete band at the top of the column and were collected after the BP eluted. The quinone mixture was eluted from silica gel with acetone and then applied to a KC_{18} reverse-phase high performance thin-layer plate (Whatman, Englewood Cliffs, NJ) and developed in methanol/benzene/water 90:5:5. [^{3}H]BP-quinones were identified by co-chromatography with authentic BP-quinone

standards and were eluted from the reverse-phase plate with benzene. Radioactivity was quantitated and the concentration of [^{3}H]BP-quinones determined.

Organ cultures containing 4 tracheas were exposed to 1 μM [^{3}H]BP or 0.7 μM [^{3}H]BP-quinones for 18 hr in 5 ml or 2 ml of culture media, respectively. The tritiated compounds were added in 10 μl of dimethyl sulfoxide. Culture dishes with no tissue but containing either [^{3}H]BP or [^{3}H]BP-quinones served as controls for spontaneous oxidation of these substrates. Metabolites were extracted by adding 2.5 ml of acetone; a 5 ml aliquot of ethyl acetate containing 0.08% butylated hydroxytoluene (to prevent further oxidation) was added to the mixture and vortexed for 2 min. Phases were separated by centrifugation at 2,900 rpm for 15 min. The organic phase was dried over anhydrous $MgSO_4$ and evaporated in a stream of N_2. The organ culture media obtained from tracheas exposed to the tritiated compounds were subjected to 3 additional organic extractions. The polar phase was then adjusted to pH 5 with 0.2 M sodium acetate buffer and then treated with 1000 units of β-glucuronidase (type b-10 from bovine liver; Sigma, St. Louis, MO) for 2 hr at 37°. The deconjugated BP metabolites were extracted into acetone/ethyl acetate, dried and evaporated by the procedure mentioned above.

High pressure liquid chromatography (HPLC) was performed on acetone/ethyl acetate extracts. Samples were redissolved in 25 μl of absolute methanol; 10 μl was injected into a custom-made high-pressure liquid chromatograph fitted with a Rheodyne 7120 injection valve, a precolumn packed with CO:PELL ODS (Whatman), and a μBondapak C_{18} column (Waters Associates, Milford, MA). Metabolites were eluted at ambient temperature at an initial flow rate of 0.8 ml per min in the reverse-phase mode by a linear gradient of methanol/water 60-80% until BP (added as a marker) eluted from the column. Authentic BP-metabolites were obtained from the Carcinogenesis Program of the National Cancer Institute and were used as UV absorbing standards. Fractions were collected at 30-sec intervals and tritium was quantitated by liquid scintillation spectrophotometry. Results were calculated by a PL/1 computer program and radioactivity distribution profiles were represented graphically by a digital plotter interfaced with an IBM 360/370 system.

RESULTS

To demonstrate that tracheal organ cultures can produce conjugates of BP-quinones with glucuronic acid starting with BP as the substrate, tracheal organ cultures were incubated with 1 μM [^{3}H]BP for 18 hr. The media was extracted a total of four times with ethyl acetate to remove organic soluble metabolites and the polar phase was exposed to β-glucuronidase. Figure 1. is an HPLC profile of the ethyl acetate soluble metabolites released by the enzymatic hydrolysis. Metabolites corresponding to a BP-polyol, 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene (7,8-diol) and BP phenols have been previously identified (9). Also present are peaks co-eluting with the BP-quinones.

A BP-quinone mixture was incubated with tracheal organ cultures in order to show definitively that the tracheal organ cultures could use BP-

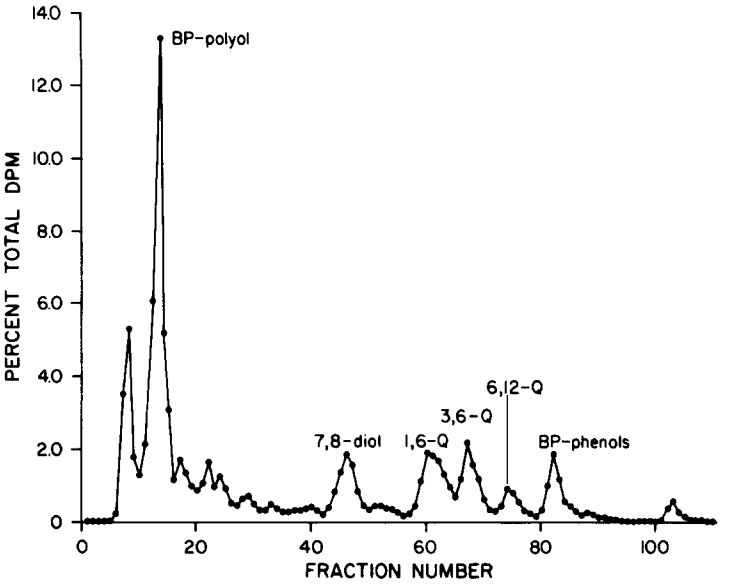

Figure 1. Tracheal organ cultures were incubated with 1μM [^{3}H]BP in 5 ml of culture medium for 18 hr. The culture medium was extracted 4 times with acetone/ethyl acetate and then exposed to 1000 units/ml of β-glucuronidase. An acetone/ethyl acetate extraction was performed and radioactivity analyzed by reverse-phase HPLC. 1,6-Q, 1,6-benzo(a)pyrene quinone; 3,6-Q, 3,6-benzo(a)pyrene quinone; 6,12-Q, 6,12-benzo(a)pyrene quinone.

quinones as a substrate for secondary metabolic processes. The chromatographic profile of BP-quinones obtained after an 18 hr incubation with and without tracheal tissue is shown in Figure 2. Radioactivity present co-chromatographs with 1,6-,3,6-, and 6,12-BP quinones. It is evident that there are considerably less BP-quinones present in the medium after incubation of the substrate with tissue than without. Since all of the radioactivity added to the culture medium was recovered after the incubation, very little, if any, was bound or adsorbed to the tissue. After the 18 hr incubation without tissue about 15% of the radioactivity became water-soluble. This value was used to normalize results in order to determine the conversion of BP-quinones to water-soluble products mediated by the tissue. After incubation of the quinone mixture with tracheal organ cultures for 18 hr about 66% of the

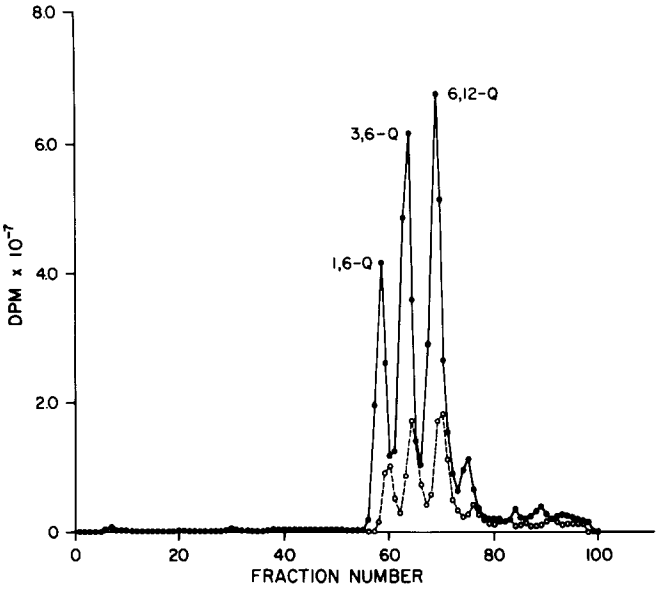

Figure 2. Organ cultures consisting of 4 tracheas were exposed to a 0.7 μM [^{3}H]BP-quinone mixture in 2 ml of culture medium for 18 hr. An acetone/ethyl acetate extract of the medium was subjected to reverse-phase HPLC, (O——O); 18 hr incubation control lacking tissue, (●——●).

organic-soluble radioactivity disappeared and became aqueous-soluble. Exposure of the aqueous-soluble radioactivity to β-glucuronidase released 30% of the radioactivity associated with this phase. Figure 3. is an HPLC profile of the acetone/ethyl acetate soluble metabolites released by β-glucuronidase treatment of the aqueous-soluble counts. Peaks chromatographing with the 3 quinones are present but the peak co-eluting with the 1,6-BP quinone appears significantly diminished. In addition, a peak representing a highly polar compound chromatographed near the origin of this radioactivity profile. The remaining 70% of the aqueous-soluble radioactivity was not sensitive to β-glucuronidase and was not identified.

DISCUSSION

There has been considerable speculation pertaining to the role of 6-OH BP in carcinogenesis by this polynuclear hydrocarbon. Ts'o et al.,

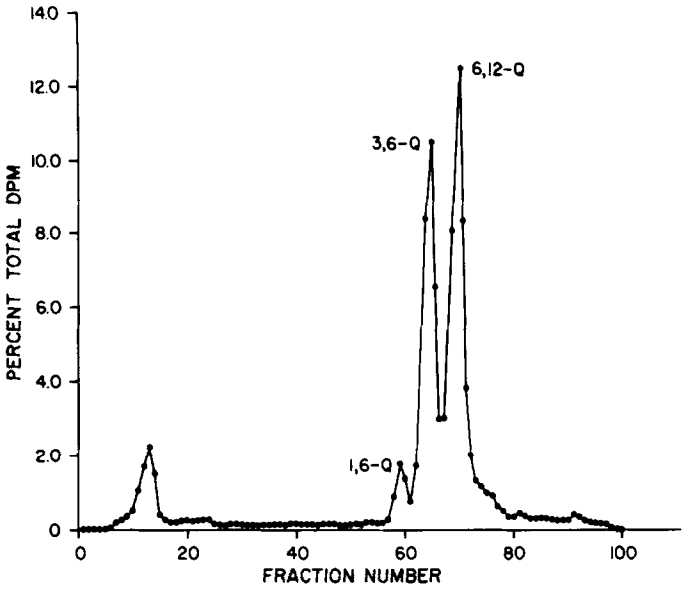

Figure 3. Organ culture media in which 4 tracheas had been incubated in the presence of 0.7 μM [^{3}H]BP-quinones for 18 hr was extracted exhaustively with acetone/ethyl acetate. The polar phase was treated with 1000 units of β-glucuronidase for 2 hr. An acetone/ethyl acetate extract was analyzed by HPLC.

(12) have shown that 6-OH BP can bind to DNA and data suggests that 6-OH BP can initiate fibrosarcomas in rats (13). 6-OH BP is a precursor to BP-quinones but the precise mechanism of quinone formation is unknown. Both enzymatic and auto-oxidative mechanisms have been proposed. Lind et al., (14) and Fahl et al., (15) have demonstrated that BP-quinones can be conjugated with UDP-glucuronic acid in the presence of microsomes. DT-diaphorase has been postulated as the reductase necessary to produce the hydroxyl group needed for linkage with glucuronic acid. Mass and Kaufman (12) observed that a large proportion of the metabolites produced by tracheal epithelial microsomes are BP-quinones. However, tracheal organ cultures produce little of these products, and the proportion diminishes with further incubation. The data presented here indicates that BP-quinones are not "end-products" of metabolism, as previously thought, but that the quinones apparently can become substrates for UDP-glucuronyl transferase

in vivo, presumably after interaction with a quinone reductase. Deconjugation with β-glucuronidase should again yield dihydroxy-BP derivatives but since these compounds are unstable in the presence of oxygen, they most probably auto-oxidize back to BP-quinones (16). The observation that most of the metabolites of BP-quinones are not sensitive to hydrolysis by β-glucuronidase indicates that they may be conjugated with other substrates (17) or poly-hydroxylated via "recycling" to water-soluble derivatives. This may partially explain our previous observation of an abundance of BP-quinones produced by tracheal microsomes in vitro and their paucity among BP metabolites produced by tracheal organ cultures.

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