OXYGENATION OF BENZO[a]PYRENE BY PLANT MICROSOMAL FRACTIONS

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1. Introduction

Benzo[a]pyrene is a highly carcinogenic compound that occurs universally [1,2]. An estimated 894 tons are released annually in the US alone, mainly in combustion processes [1].

BP is extensively metabolized in mammalian systems, in particular by the mixed-function oxygenase system of liver microsomes [3]. Species of cytochrome P450 are involved in this process and lead via epoxide intermediates to various phenolic and dihydrodiol metabolites. A defined diol-epoxide is though to act as the ultimate carcinogen by binding covalently to cellular DNA [3]. BP is a relatively stable compound [1,2] which is, for example, completely inert under waste treatment conditions [4] but it has been found to be metabolized by certain microorganisms [5], by intact plants [6,7] and by cultured plant cells [8,9]. The biochemical reactions leading to the various plant metabolite fractions of BP are as yet unknown although certain analogies with the liver microsomal system have been postulated on the basis of HPLC data [10].

It is now reported that plant microsomal fractions catalyze the oxygenation of BP and that this process differs considerably from the oxygenation of BP by liver microsomal fractions.

2. Experimental

2.1. *Materials*

Cell suspension cultures of parsley (*Petroselinum hortense* Hoffm.) and soybean (*Glycine max* L.) were grown as in [9]. Microsomal fractions were obtained by the ultracentrifugation or Mg^{2+}-precipitation procedures [11], both preparations giving identical results. The growth of pea seedlings (*Pisum sativum* L.) and the preparation of the microsomal fraction from the primary leaves of pea have also been described [12]. [7,10-^{14}C]BP was purchased from Amersham-Buchler, Braunschweig. The authentic 1,6-, 3,6- and 6,12-quinones of BP were prepared according to [13] and purified by silica gel chromatography and HPLC (see below). They were formed in relative amounts of 51:30:19.

Abbreviations: BP, benzo[a]pyrene. SDS, sodium dodecylsulphate. DMF, dimethylformamide. DMSO, dimethylsulfoxide. HPLC, high-pressure liquid chromatography

2.2. *General procedures*

Determinations of radioactivity [9,12] and of protein content [14] have been described. Thin-layer chromatography was carried out on precoated silica gel G plates (Merck no. 5554) using the following solvent systems: (A) benzene; (B) benzene/ethanol, 95:5 (v/v); (C) benzene/ethanol, 9:1 (v/v). The solvent system used for paper chromatography [15] was (D) butan-1-ol/acetic acid/water, 2:1:1 (by vol.)

2.3. *Incubation procedure*

All steps involving BP were carried out under red light ($\lambda \geqslant 550$ nm). A solution of [^{14}C]BP (58 nmol, 90 000 dpm) in DMSO (10 μl) was pipetted into an incubation tube and frozen (0°C). The microsomal suspension (3 mg protein in 490 μl 50 mM Tricine–KOH, pH 7.5) was added, followed by thorough mixing at 25°C and aerobic incubation in the dark (60 min, 30°C, shaking in water bath). The incubation was terminated by addition of 200 μl acetone and 1 ml ethyl acetate. The organic phase was isolated, followed by re-extraction of the aqueous phase with five 1-ml portions of ethyl acetate. Control incubations were performed with microsomes heated at 100°C for 10 min at 10 mg protein/ml.

2.4. *Isolation of metabolites*

The pooled organic extracts were concentrated and applied to a column (12 X 1.8 cm) of silica gel 60 (Merck no. 7754) packed in solvent system A. Thorough washing with solvent system A led to the removal of unchanged BP. The metabolite fraction was eluted with solvent system C, and further purified by HPLC (fig.1).

3. Results

3.1. *Effect of hydrocarbons on parsley cinnamic acid 4-hydroxylase*

The plant microsomal cinnamic acid 4-hydroxylase depends on a species of cytochrome P450 and resembles the liver microsomal oxygenase system in some other respects as well (reviewed in [16,17]).

The following hydrocarbons (at up to 500 μM) were tested as inhibitors of standard cinnamic acid 4-hydroxylase incubation mixtures derived from light-induced parsley cells [11,15]: BP, 20-methylcholanthrene, and 7,12-dimethylbenzanthracene. No inhibition above that given by the solubilyzing solvent itself was observed. DMSO or *N*-methylpyrrolidone were used at final concentrations of 10% (v/v) and inhibited by ~25%.

3.2. *Isolation and characterization of BP metabolites*

3.2.1. BP quinones

Microsomal fractions were prepared from cultured soybean cells and from primary leaves of pea and incubated with [^{14}C]BP under various conditions. These experiments which are not detailed, indicated that a polymeric and a soluble BP metabolite fraction were formed and that addition of NADPH was not required for these reactions. For identification of the soluble metabolite fraction the organic extracts from 90 parallel incubations with pea microsomes were pooled and fractionated on a column of silica gel (see section 2). About 7.5% of the initial amount of radioactivity was recovered in the metabolite fraction and was further fractionated by reversed-phase HPLC [3,18] on a column which had been pre-calibrated with authentic BP and BP-quinones. The applied radioactivity appeared up to >90% in the area of BP-quinones (fig.1). The data of fig.1 as well as analytical HPLC runs of higher resolution (not shown) indicated that 42% of the radioactivity appeared in the position of the authentic BP-1,6-quinone (peak I), 38% appeared as the BP-3,6-quinone (peak II) and 21% as the BP-6, 12-quinone (peak III). The individual quinone fractions were re-chromatographed under the conditions described in fig.1 and were further characterized as follows.

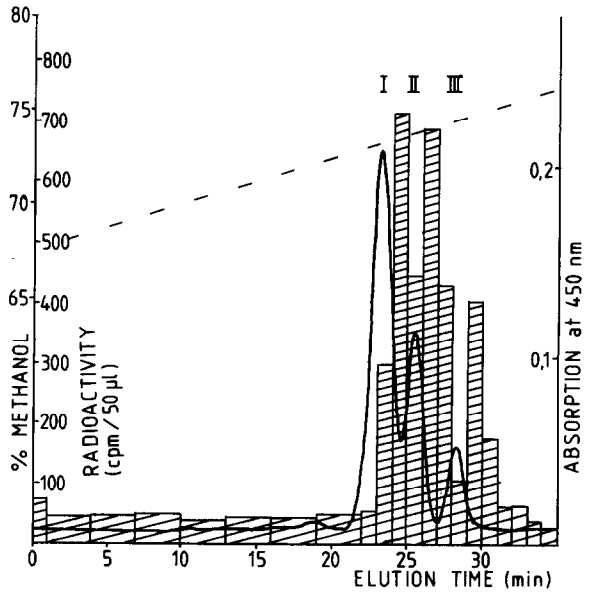

Fig.1. Fractionation of BP metabolites by HPLC. A Waters instrument was used with a column (30 X 0.4 cm) of μ-Bondapak C_{18} packed in 68% (v/v) aqueous methanol. About 6 μg metabolites was applied in 10 μl solvent system C, followed by washing of the column with 68% methanol before elution by the indicated (– – –) linear gradient of methanol in water. The flow rate was 0.5 ml/min with continuous recording of A_{450} (——). Fractions were collected every 3 min or 1 min (see bars) and their radioactivities determined on 50 μl aliquots (indicated by bars). Relative to the cuvette for optical recording, the fractions appeared at the outlet with a time lag of ~1.5 min. The quinone part of the HPLC profile is shown and the 3 peaks discernible are labeled I, II and III.

Peaks I and II had main fluorescence excitation maxima at 402 nm and 466 nm, respectively, and main emission maxima at 455 nm and 570–580 nm, respectively. Peak III was non-fluorescent (data obtained in ethanol on a Perkin-Elmer MPF 21 Fluorescence Spectrophotometer). The UV-spectra of peaks I, II and III are shown in fig.2a. Treatment of the quinone fractions with sodium borohydride [22] led to drastic color changes (dark-blue from yellow with peaks I and III and violet from red with peak II). The resulting absorption spectra are shown in fig.2b. The various spectral data obtained were in agreement with the spectral data in [19–21] for BP-1,6-quinone

(peak I), BP-3,6-quinone (peak II) and BP-6,12-quinone (peak III) or the derived semi-quinones [22].

The spectra in fig.2 were also obtained in independent experiments where the BP-metabolites were purified by repeated 2-dimensional thin-layer chromatography rather than HPLC (not shown).

The characterization of the BP-metabolites formed by soybean microsomes led to identical results although HPLC analysis indicated that in this case unidentified polar BP-metabolites, possibly polyhydroxylated compounds, were formed in addition to the three isomeric quinones.

The amounts of BP-quinones formed were ~1.5 nmol/mg protein with soybean microsomes and 3 nmol/mg protein with pea microsomes (60 min values). Less than 10% of these amounts were formed by the heat-inactivated control microsomes.

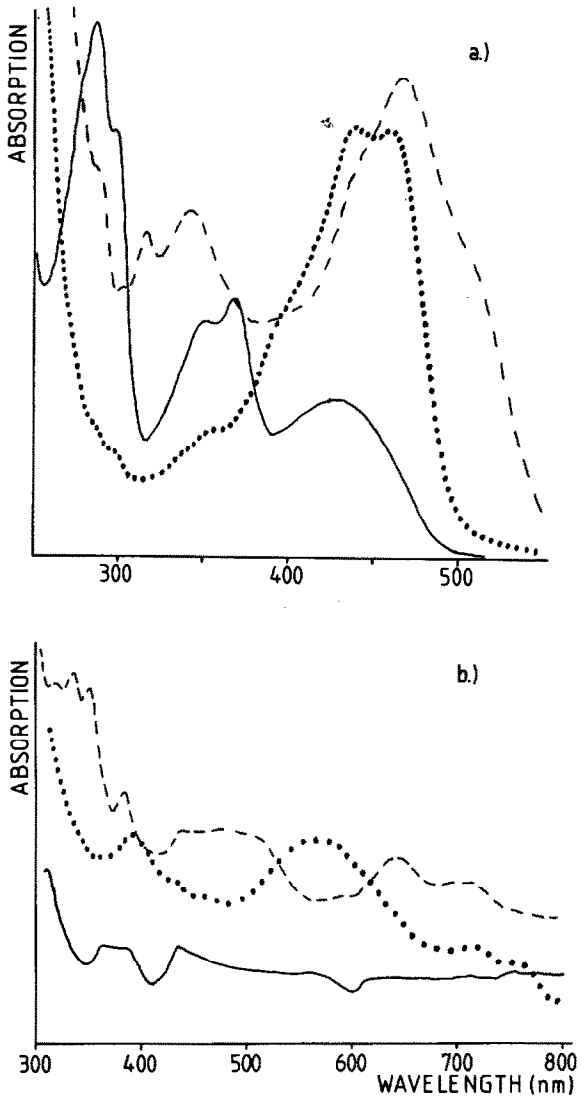

Fig.2. (a) UV spectra of aliquots of peak I (. . .) peak II (– – –) and peak III (——) of the HPLC profile in fig.1. Spectra were obtained in 90% methanol by use of a Perkin Elmer 554 spectrophotometer (arbitrary units). (b) Visible absorption spectra of the isolated quinone metabolites after borohydride treatment according to [22]. Symbols are as in fig.2a. The spectra were obtained in 50% DMSO (arbitrary units).

3.2.2. Glutathione conjugate(s)

Addition of 2 mM glutathione to the incubation mixture in section 2 led to the appearance of a glutathione-conjugate fraction which was detected by paper chromatography in solvent system D. Similar results were reported in [12]. Incubation with [^{14}C]-BP-quinones also led to a glutathione-conjugate product, even when heat-inactivated microsomes were employed (not shown).

3.2.3. Polymeric products

A considerable amount of [^{14}C]BP was incorporated into a chromatographically immobile metabolite fraction (solvent systems A–D) by soybean as well as by pea microsomes. This product amounted to ~50% of the quantities of quinones formed (see above). After chromatography in solvent system D, most of the material at the origin could be eluted with either 2% (w/v) SDS (2 X 1 h, 100°C) or with DMF (3 X 2 h, 150°C). When the SDS extract was examined by SDS gel electrophoresis [9] >85% of the radioactivity failed to enter the gel. Analysis of the DMF-extract on a column of Sephadex LH-60 (packed in DMF; [22]) revealed a broad M_r distribution of 1000–25 000 (in comparison with defined polystyrol standards).

4. Discussion

The above results reveal considerable differences between the metabolism of BP by microsomal fractions from plants versus those from liver.

(1) BP and two other polycyclic hydrocarbons were non-inhibitory in the plant microsomal cinnamic acid 4-hydroxylase reaction even though this reaction depends on a species of cytochrome P450 [16,17].

(2) NADPH was not required in the plant microsomal oxygenation of BP, but it is an obligatory cofactor in the liver system [3].

(3) The isomeric 1,6-, 3,6- and 6,12-quinones of BP were identified as the predominant BP metabolites of pea and soybean microsomes whereas the epoxide-derived phenolic and dihydrodiol derivatives of BP are characteristic for the liver system [3].

BP-quinones are formed only as a minor metabolite fraction by liver microsomes [3] and by purified liver cytochrome P450 [23].

Studies with liver microsomes [19,20,24] and with sheep seminal vesicles [25,26] have shown that the 1,6-, 3,6- and 6,12-quinones of BP can arise via the easily autoxidizable 6-hydroxy-BP by reactions which do not involve cytochrome P450. This pathway may also exist in plant microsomes because the % distributions of the 3 quinone fractions formed by soybean and pea microsomes were similar to the % distribution observed in the chemical oxidation of BP (see section 2) and to the % distribution reported in [20] for the autoxidation of 6-hydroxy-BP. The 6-hydroxy-BP radical has furthermore been shown to be chemically reactive and to bind covalently to polynucleotides [19]. The isomeric BP-quinones form conjugates with glutathione (see above), or they can be reduced and then conjugated with, for example, D-glucuronic acid [27,28]. If these various observations are applied to the present results, the metabolic scheme in fig.3 is obtained.

The BP-quinones possess low or zero mutagenicity against microbial [26,29] or mammalian cells [30] although they are cytotoxic [31]. The formation of glutathione conjugates and polymeric metabolites (see above) as well as the enzymatic co-polymerisation of BP-quinones into lignin (unpublished) may further contribute to the detoxification of BP by plant enzymes.

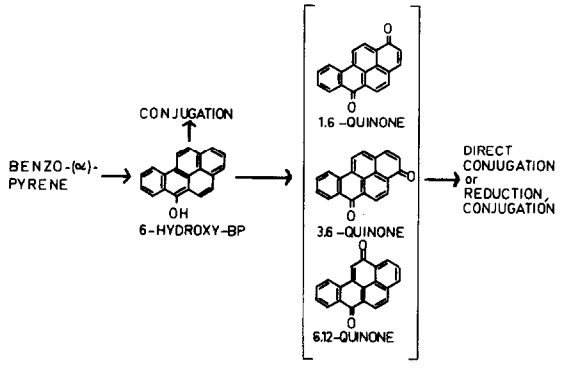

Fig.3. Proposed pathway for the oxygenation of benzo[a]-pyrene by plant microsomes.

Acknowledgements

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