INCORPORATION OF BENZO[α]PYRENE QUINONES INTO LIGNIN

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1. Introduction

The carcinogen benzo[α]pyrene (BP) is found as a universal environmental contaminant. Its typical level in food plants amounts to several μg/kg dry wt [1,2]. BP has been found to be metabolized by intact plants [3,4] and cultured plant cells [5,6]. The BP metabolite profiles of cultured *Chenopodium* cells and of liver have been compared [7]. Cultured soybean cells formed a multitude of BP-metabolites among which the BP-3,6-quinone and several polymeric fractions have been chemically characterized [6,8].

In [9], plant microsomal fractions were found to catalyze the conversion of BP to the 3 isomeric BP-quinones. Here, we describe a pathway for further detoxification of BP, namely the incorporation of the BP-quinones into lignin.

2. Experimental

2.1. *Materials*

The source of [7,10-^{14}C]BP, the preparations of the mixed BP-quinones and of a pea microsomal fraction as well as the general procedures have been described [9]. The coniferyl and vanillyl alcohols were obtained from Fluka (Neu-Ulm). Horse radish peroxidase (grade I) was purchased from Boehringer (Mannheim).

2.2. *Co-polymerization of BP-quinones*

The following representative procedure was adopted from the method in [10], all steps being performed under nitrogen atmosphere and red light ($\lambda \geq 550$ nm). Solutions of:

(a) 1.69 g (6 mmol) [^{14}C]BP-quinones (68.5 μCi) in dimethylsulfoxide;
(b) 2.16 g (12 mmol) coniferyl alcohol and 56 mg peroxidase;
(c) 0.46 g hydrogen peroxide were pumped over a period of ~60 min into a well-stirred solution of 38 mg vanillyl alcohol and 19 mg peroxidase.

The aqueous buffer used in all solutions was 10 mM sodium phosphate (pH 7.5) deaerated by vacuum filtration through a 0.4 μm millipore filter and subsequent flushing with N_2 (1 h). An Abimed Minipulse 2 peristaltic pump was used for solutions (b) and (c), while solution (a) was added via teflon tubing under constant hydrostatic pressure at the same rate as the pumped solutions. After an additional incubation time of 35 min the precipitated reaction product was isolated by low-speed centrifugation. It was washed 3 times with deaerated water followed by up to 40 extractions with ethyl acetate until the washing and DMF test solutions of the polymer were free of monomeric BP-quinones (assayed by thin-layer chromatography in solvent systems B and C of [9]). The final polymeric product (1.7 g) was dissolved in 20 ml DMF for further analysis.

2.3. *Co-polymerization of BP*

The polymerization was performed using two reaction chambers because BP required oxidation by pea microsomes before being incorporated into lignin.

Chamber 1: A suspension of 75 mg pea microsomal protein [9] in 15 ml 100 mM Na–tricine (pH 7.5) and a solution of 28.1 μmol [^{14}C]BP (0.25 μCi) in 0.8 ml dimethylsulfoxide were pumped into reaction chamber 1 at flow-rates of 13.3 ml/15 min and 0.75 ml/15 min, respectively.

Chamber 2: The content of chamber 1 (flow rate, 14 ml/15 min) as well as solutions of: (a) 69.5 μmol coniferyl alcohol and 0.66 mg peroxidase in

Abbreviations. BP, benzo[α]pyrene; DMF, dimethylformamide

3 ml 10 mM sodium phosphate (pH 7.5); and (b) 69.5 μmol hydrogen peroxide in 10 mM sodium phosphate (pH 7.5) (flow rate, 2.6 ml/15 min) were pumped simultaneously into reaction chamber 2 which contained the following well-stirred receiving solution (0.22 mg vanillyl alcohol and 0.22 mg peroxidase in 3 ml 10 mM sodium phosphate (pH 7.5)). The phosphate buffer was deaerated as in section 2.2. Oxygen was excluded from chamber 2 by flushing with a stream of nitrogen and all operations were performed under red light ($\lambda \geq 550$ nm). The entire pumping operation took 17 min and total incubation time was 160 min. The precipitated reaction product was isolated by low speed centrifugation and washed 3 times with deaerated water. The precipitate was then extracted with ethyl acetate in order to remove monomeric quinones (see section 2.2). The resulting residue was extracted with DMF (25°C) to give DMF-extract 1 (9.9 μg BP incorporated to 0.06 mol%). Since most of the material did not dissolve in DMF, the residue was suspended in 1 ml DMF, frozen in liquid N_2 and pulverized by means of a Mikro-dismembranator instrument (Braun Melsungen no. 330A, 5 × 1 min). The material was then extracted with three 10 ml portions of DMF (150°C, 5–20 h) to give DMF-extracts 2,3 and 4. Their BP-contents were as follows, 23 μg BP at 0.13 mol% (extract 2), 92 μg BP at 0.52 mol% (extract 3) and 177 μg BP at 1 mol% (extract 4). Between DMF-extractions 3 and 4 the residue was treated with 10 ml 1% (w/v) aqueous sodium dodecylsulphate (100°C, 5 h). This treatment did not result in solubilization of radioactivity. The DMF-extracts 1–4 were reduced to ~3 ml each by means of a rotary evaporator.

3. Results

3.1. *Use of BP-quinones*

The mixed [^{14}C]BP-quinones were prepared by chemical synthesis [9]. They were then employed in a recent version [10] of the original Freudenberg methods [11] to produce artificial lignin. The insoluble reaction product was isolated and purified (see section 2.2). In preliminary experiments employing various relative amounts of the [^{14}C]BP-quinones, up to 8.3 mol% of the polymeric product consisted of incorporated BP. In the preparative incorporation experiment

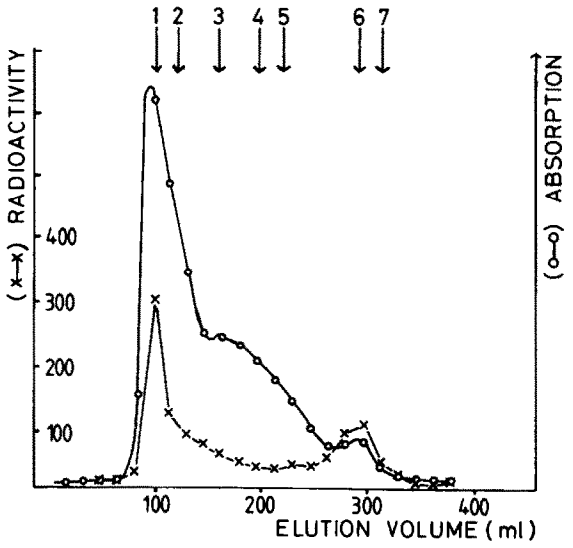

Fig.1. Chromatography of a [^{14}C]BP-quinone–lignin copolymer on a column (110 × 2 cm) of Sephadex LH-60. About 10 mg of the reaction product of section 2.2 was applied to the column in a small amount of DMF, followed by development in DMF. The elution was monitored at 280 nm (o——o, arbitrary units). Fractions of 8 ml were collected and their radioactivity was determined (x——x, cpm/ml). In separate experiments the column was calibrated with defined polystyrene standards (from Waters Associates; see arrows in the upper part of the graph). These standards had the following stated molecular weights: (1) 35 000 (exclusion volume); (2) 17 500; (3) 8500; (4) 4000; (5) 2900; (6) 680; (7) position of coniferyl alcohol, M_r 180.

of section 2.2 the incorporation was 1.4 mol%. The molecular weight distribution of the artificial lignin was examined in DMF-solution on a column of Sephadex LH-60 pre-calibrated with polystyrenes of defined molecular weight (fig.1). The elution profiles of UV-absorption (reflecting mainly lignin) and of radioactivity (reflecting incorporated BP) were similar. Most of the product was $> M_r$ 5000. A UV-spectrum which was typical for the fractions M_r 5000–20 000 is shown in fig.2. It was similar to the UV-spectra of an artificial lignin prepared in the absence of BP-quinones and of spruce lignin (fig.2).

In control experiments the [^{14}C]BP-quinones by themselves were found to polymerize spontaneously in a reaction which did not require peroxidase. The product consisted primarily of species of M_r ~700 (determined on Sephadex LH-60, not shown). However, even when fractions of comparable molecular weight were used, the UV-spectrum of the control polymer differed clearly from the lignin spectra (fig.2).

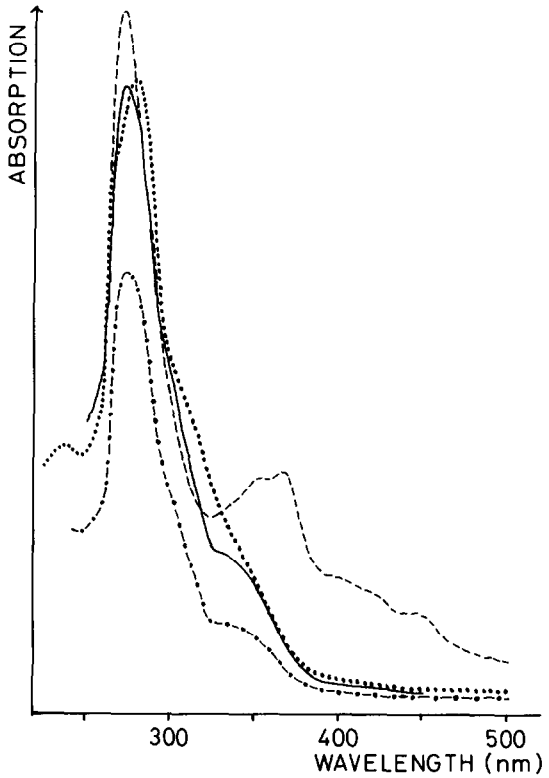

Fig.2. Ultraviolet spectra of polymer samples. Spectra were taken in DMF solution on a Perkin Elmer model 554 spectrophotometer (arbitrary units): (——) spectrum of the polymer fractions of M_r ~8000 of fig.1; (–.–) spectrum of a polymer sample corresponding to that of previous curve, with sample prepared according to section 2.2 in the absence of BP-quinones; (– –) spectrum of a control polymer of M_r ~8000 which was prepared according to section 2.2 omitting peroxidase and coniferyl alcohol; (. . .) spectrum of an authentic Björkman-type spruce lignin.

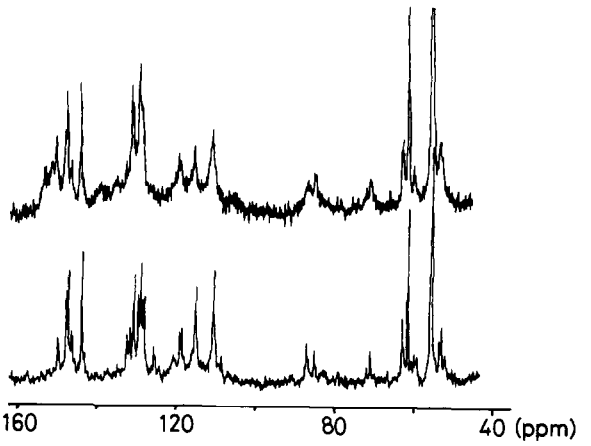

Fig.3. ^{13}C NMR spectra: upper spectrum of M_r ~20 000 fractions of fig.1; lower spectrum of corresponding polymer fractions which were prepared in the absence of BP-quinones. The spectra were taken in deuterated dimethylsulfoxide with tetramethylsilane as an internal standard. A Bruker WP-80 instrument (20.15 MHz) was used at 36°C in the Fourier transform mode.

The [^{14}C]BP-quinone–lignin fractions of M_r ~20 000 (see fig.1) were further characterized by ^{13}C NMR spectroscopy (fig.3). The incorporation of BP-units was not high enough to detect peaks stemming from the BP-quinones but the comparison with the ^{13}C NMR spectrum of a synthetic lignin (fig.3) as well as with published ^{13}C NMR spectral data for lignins [12,13] clearly identify the [^{14}C]BP-copolymer as a lignin derivative.

3.2. *Use of BP*

When [^{14}C]BP instead of the [^{14}C]BP-quinones was added to the incubation mixture of section 2.2 the ^{14}C-incorporation into the lignin product was insignificant. In further experiments [^{14}C]BP was pre-

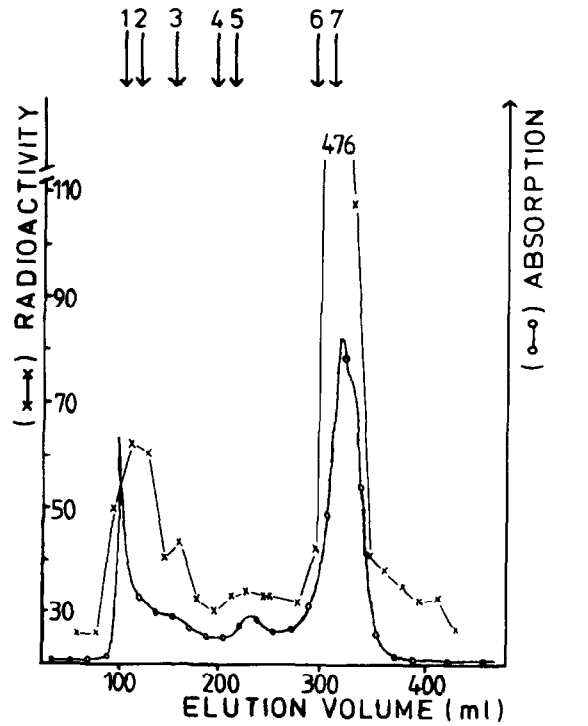

Fig.4. Chromatography of the DMF-extract 4 of section 2.3 on a column of Sephadex LH-60. The conditions and symbols are as in fig.1.

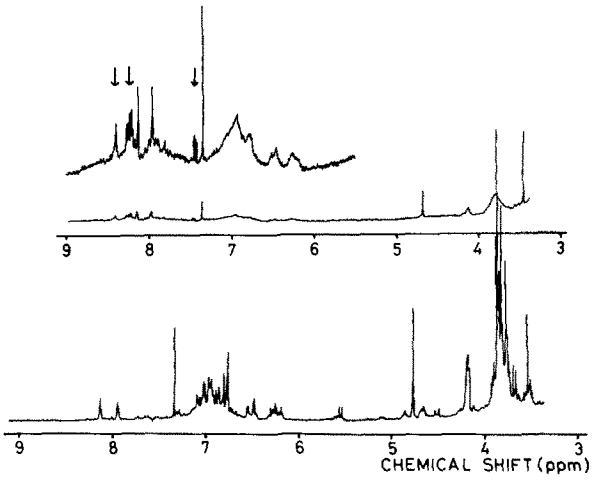

Fig.5. ^{1}H NMR spectra: upper spectrum of the fractions of M_r ~8000 of fig.4 (taken in deuterated acetone/deuterated dimethylsulfoxide, 75:25 (vv)); lower spectrum of a polymer fraction of M_r ~1500 which was prepared in the absence of BP-quinones according to section 2.2 followed by chromatography as in fig.1 (spectrum taken in deuterated acetone). A Bruker WM 250 instrument (250,10 MHz) was used at 23°C with tetramethylsilane as an internal standard. About 15 000 individual scans were accumulated in order to obtain the spectra shown.

incubated with a pea microsomal fraction and this incubation mixture was then pumped into the lignin-forming incubation mixture. About 1 mol% BP was now incorporated into the lignin product under the conditions detailed in section 2.3. Gel chromatography on Sephadex LH-60 indicated that the UV-absorption and the radioactivity both mainly appeared near M_r 700 (fig.4). The UV-spectra of various column fractions appeared to be composed of both the lignin and the BP-quinone control polymer spectra of fig.2. A ^{1}H NMR spectrum of the material corresponding to M_r ~8000 is shown in fig.5. The comparison with the ^{1}H NMR spectrum of a synthetic lignin (fig.5) as well as with published ^{1}H NMR spectral data for lignins [13,14] ascertained the lignin nature of the [^{14}C]BP-containing material. In addition, several minor peak multipletts were present near 8.4, 8.25 and 7.45 ppm. Peak multipletts of similar chemical shifts have been reported for a number of BP-derivatives [15].

4. Discussion

The above data establish that [^{14}C]BP-quinones but not BP itself could be polymerized into a product which appeared to be lignin on the basis of UV, ^{1}H NMR and ^{13}C NMR spectra. The incorporation of radioactivity from [^{14}C]BP into a lignin-like cell fraction has been observed in cultured soybean cells [6,8]. An artefactual polymerization reaction of the chemically reactive BP-quinones is also described above and may have occurred as a side reaction of the oxygenation of BP by plant microsomal fractions [9].

The synthetic lignin and the incorporated BP-species showed similar molecular weight distributions (fig.1,4). This is expected from the spontaneous and essentially random mechanism of lignin formation which proceeds by polymerization of radical and quinone methide intermediates [11,13]. Foreign chemicals may react with these lignin intermediates and thus be co-polymerized. In addition to the BP-quinones 4-chloroaniline and 3,4-dichloroaniline have also been shown to readily copolymerize into artificial lignin (unpublished). Furthermore, an incorporation into lignin-like residues has often been observed in in vivo studies of the plant metabolism of pesticides although the chemical characterization of such residues has been difficult [16]. A polymeric metabolite fraction containing covalently bound 2,4-dichlorophenoxyacetic acid and its 4-hydroxy-derivative have been characterized as being primarily lignin (D. Scheel, H. S. unpublished).

The incorporation of foreign chemicals into lignin may represent an important mechanism for detoxification since lignin is the second most abundant natural polymer [13].

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