Processing of Bisphenol A by Plant Tissues: Glucosylation by Cultured BY-2 Cells and Glucosylation/Translocation by Plants of *Nicotiana tabacum*

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Bisphenol A (BPA, 4,4'-isopropylidenediphenol), an endocrine disrupter with estrogenic properties, was supplied to tobacco BY-2 cells in suspension culture and the chemical nature of its metabolites was investigated. The concentration of BPA in the culture medium decreased rapidly and became undetectable at 2.5 h after the application. Four metabolites of BPA were observed in a methanol extract of the cells when the culture was supplemented with [14C]BPA. The most abundant metabolite was determined to be 4,4'-isopropylidenediphenol-O-β-D-glucopyranoside (BPAG) by mass spectrometry, nuclear magnetic resonance spectroscopy and by hydrolysis with β-glucosidase. This identification was confirmed by synthesis. When [14C]BPA was administrated to tobacco seedlings from their roots, radioactivity was incorporated in BPAG and three unidentified metabolites. These metabolites were accumulated in the leaves after 4 h exposure, indicating that tobacco seedlings absorbed BPA through their root systems, metabolized to its β-glucoside and translocated the metabolites to their leaves.

**Keywords:** Bisphenol A — BY-2 cells — Glucoside — *Nicotiana tabacum* — Phytoremediation.

Abbreviations: BPA, bisphenol A (4,4'-isopropylidenediphenol); BPAG, 4,4'-isopropylidenediphenol-O-β-D-glucopyranoside; NMR, nuclear magnetic resonance; FAB-MS, fast atom bombardment mass spectrometry; MSD, Murashige-Skoog medium containing 16 µM of 2,4-D

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**Introduction**

Various chemical compounds have been discharged into terrestrial and aquatic environments with our industrial activities. Some of them with a hormonal activity are called endocrine disrupters. They have been implicated in the abnormal sexual development of reptiles and birds (Guillette et al. 1994, Guillette et al. 1995, Fry 1995), abnormal courtship behavior of male fish and the attenuation of sperm production in mammals (White et al. 1994, Jobling et al. 1995, Carlsen et al. 1992, Auger et al. 1995, Baatrup and Junge 2001). The estrogenic properties of bisphenol A (BPA), known since the early work of Dodds (Dodds and Lawson 1938), have recently been a cause for anxiety because of the environmental release of the compound (Staples et al. 1998). BPA is used in the manufacture of polycarbonate and epoxy resins, and as a stabilizer or antioxidant for many types of plastics such as polyvinyl chloride (Ash and Ash 1995). Annual world production of this compound has been estimated at more than 500,000 tonnes (Staples et al. 1998).

Significant amounts of BPA have contaminated terrestrial and aquatic environments (Alexander et al. 1988). Dorn et al. (1987) reported that BPA in river water was degraded within 4 d and a bacterium responsible for the breakdown was isolated (Lobos et al. 1992). This bacterium (MV1) degraded BPA to 4-hydroxybenzoic acid by successive hydroxylations and oxidations (Spivack et al. 1994). However, the use of this bacterium to degrade BPA in polluted sites might have an adverse ecological impact through unlimited propagation. Plants also have been considered as suitable agents for the biodegradation of lipophilic compounds (Cunningham and Ow 1996) and would probably be more easily controlled in propagation. However, the ability of plant cells to take up and metabolize BPA has not been investigated. In this study, we exposed a culture of tobacco BY-2 cells to BPA and studied the ability of the cells to take up and metabolize the compound. The chemical structure of its metabolites was also investigated.

**Results**

**Uptake and metabolism of BPA by BY-2 cells**

To determine whether plant cells can absorb BPA, tobacco BY-2 cells in suspension were exposed to 10 mg liter$^{-1}$ of BPA in the medium. The BPA concentration in the medium rapidly decreased to one-third of the initial value after 0.5 h, and to total disappearance by 2.5 h (Fig. 1A). In the absence of cells there was no reduction in the concentration of BPA. The concentration of BPA also decreased when the culture medium was replaced by a freshly prepared one. The rate of decrease depended upon the number of cells in the culture (data not shown). These results indicate that BY-2 cells have the ability to take up BPA and BPA is not adsorbed by culture vessels or substances secreted by the cells.
Fig. 1  (A) Concentration of BPA in culture medium in the presence and absence of BY-2 cells in suspension culture. BPA was supplemented to a final concentration at 10 mg liter\(^{-1}\) and incubated at 25°C. The amount of BPA in the culture medium was determined by HPLC at indicated times. Filled circles, BPA was added to cultured cells; open circles, cells were collected on a filter paper and re-suspended to freshly prepared MSD, then BPA was added; filled triangles, cells were removed by filtration and BPA was added to the filtrate; open triangles, BPA was supplemented into freshly prepared MSD. Vertical bars indicate standard deviations (\(n = 3\)). (B) Radioactivity in freshly prepared culture medium with or without BY-2 cells in suspension culture. [\(^{14}\)C]BPA was supplemented into 2-week-old BY-2 suspension cultured cells (filled circles) or freshly prepared MSD (open circles). The radioactivity of 25 \(\mu\)l of the medium was then determined by liquid scintillation counting at indicated times. Vertical bars indicate standard deviations (\(n = 3\)).

Fig. 2  Radiochromatogram of methanol extracts of BY-2 cells after administration of [\(^{14}\)C]BPA. Culture of BY-2 cells were administrated with 410 kBq of [\(^{14}\)C]BPA and then incubated. The culture was sampled at 0 h (A), 0.25 h (B) and 0.5 h (C) after the start of incubation. They were extracted with methanol and 50 \(\mu\)l of the methanol-soluble fractions was analyzed by HPLC.

Fig. 3  Radiochromatogram of a medium of BY-2 suspension culture after administration of [\(^{14}\)C]BPA. BY-2 cells were administrated with 410 kBq of [\(^{14}\)C]BPA and then incubated. The culture was sampled at 0 h (A) and 2 h (B) and 20 \(\mu\)l of them were analyzed by HPLC.
To determine whether BY-2 cells metabolize BPA, radiolabeled BPA was added to the medium and cells so exposed were extracted with methanol and the extract analyzed by HPLC. Radioactivity in the medium decreased to one-third of the original level after 2 h of incubation, but it did not completely disappear (Fig. 1B). In the radiochromatogram of the methanol extract of the cells, BPA was eluted at a retention time of 37 min and four metabolites were apparent. These metabolites were coded C, D, G and L in order of elution from the C-18 column (Fig. 2C). The metabolite L appeared within 0.25 h of supplementation by \[^{14}\text{C}]\text{BPA}\) (Fig. 2B) and its concentration increased thereafter (Fig. 2C). The concentrations of the other three hydrophilic metabolites also increased during this time, but their amounts were always less than that of L. The radiochromatogram of the culture medium revealed that the concentration of BPA in the medium much decreased during the 2-h incubation (Fig. 3). In contrast, the concentration of L increased markedly and the concentrations of metabolites D and G also gradually increased in the medium during the incubation time. These results demonstrate that BY-2 cells metabolized BPA to at least four compounds during a 0.5-h incubation period, and that one compound, L, was immediately released from the cells into the culture medium.

As L was the major metabolite of BPA, it was isolated by repeated HPLC. The isolated quantity of metabolite L, although small (approximately 100 µg), was adequate for its identification by spectroscopic methods and enzymatic hydrolysis. Its identity was confirmed by synthesis and the availability of synthetic material allowed testing of the estrogenic properties of L and a comparison with those of BPA. Full details of the synthesis and estrogenic testing will be published elsewhere. Metabolites C, D and G were not identified. However, C had the same chromatographic retention time as BPA–di-O-β-D-glucopyranoside.

**Identification of metabolite L**

The \(^1\text{H}-\)nuclear magnetic resonance (NMR) spectrum of L consisted of a singlet (δ1.486, 6H) attributable to the isopropylidene methyl groups, four ortho-coupled aromatic resonances (2H each) showing that BPA was mono-substituted, two sets of doublets (δ3.591 and 3.770) showing both geminal and vicinal coupling that were readily assigned to the hydroxymethyl group of a sugar system, a complex multiplet (4H) at δ3.32–3.47, and a doublet (1H, \(J = 7.5\text{Hz}\)) at δ4.940 (Fig. 4). Taken together the signals at δ3.32–4.940 were strongly indicative of the presence of a single hexose residue. Of particular diagnostic value was the doublet at δ4.940 which was readily attributable as the anomeric proton of a β-glycoside (Collins and Ferrier 1995). Further evidence that metabolite L was BPA–mono-O-β-D-glucopyranoside was provided by Fast atom bombardment mass spectrometry (FAB-MS), which indicated a molecular weight of 390, and by hydrolysis with β-
metabolite L as BPA-mono-glucoside. Furthermore, the identification of L was isolated to allow the determination of its specificity. Insufficient L was isolated to allow the determination of its specific rotation. However, the unlikelihood of D-glucose occurring in natural systems together with the specificity of the glucosidase allow the identification of L as 4,4’-isopropylidenediphenol-mono-O-β-4-glucopyranoside (BPAG) (Fig. 4).

**Uptake and metabolism of BPA by tobacco seedlings**

To investigate whether tobacco plants have the ability to take up and metabolize BPA, tobacco seedlings were exposed to [14C]BPA through their root systems. Sixteen percent of radioactivity in the medium rapidly disappeared within 0.5 h and then the remaining radioactivity gradually decreased to 73% at 4 h (Fig. 6A). The largest part of radioactivity in the seedling was detected in roots at 0.5 h and the radioactivity in the roots gradually increased with further incubation (Fig. 6B). The amount of radioactivity incorporated into leaves linearly increased during the incubation period, its level becoming similar to that of roots at 4 h. A small amount of radioactivity was detected in stem tissue throughout the incubation period. These results suggest that tobacco seedlings can take up BPA from their root system and translocate it to their leaves.

Methanol extracts of roots, stems and leaves were analyzed by HPLC. In the root, a high amount of radioactive BPA was detected and a significant amount of radioactivity was incorporated into BPAG, metabolites C and G at 0.5 h (Fig. 7A), but the total amount was only 30% of the radioactivity which decreased in the medium. The level of BPA was decreased with longer incubation. The levels of metabolite C and BPAG did not change significantly during the 4 h incubation. The level of metabolite G increased 5-fold at 4 h. In stem tissue small amounts of BPA and three metabolites were detected only at 4 h (data not shown).

To test whether tobacco roots release metabolites of BPA into the medium, composition of radioactive materials in the incubation medium was analyzed by HPLC. A small amount of BPAG and C were detected in the incubation medium at 4 h. Each level was 15% of the radioactivity incorporated into the seedlings (data not shown). This indicates that most of the metabolites of BPA were kept in the seedlings.

In the leaf, only a little amount of BPA was detected after 0.5 h of exposure. However, considerable quantities of radioactive C, G, BPAG and BPA were detected at 1 h and the level of BPAG was increased 4-fold at 4 h (Fig. 7B). After 4 h of incubation, the amount of BPA extensively declined. These results indicate that tobacco seedlings absorbed BPA through their root systems, metabolized it to BPAG and compounds C and G, and then translocated these metabolites to their leaves.

**Discussion**

Tobacco BY-2 cells in suspension culture absorbed BPA and metabolized it to its β-glucoside (BPAG) and to three other compounds (C, D, G). The radiochromatogram of methanol extracts of the cells indicated that accumulation of BPAG started prior to an increase in concentration of the other compounds (Fig. 2B). This suggests that BPA was directly metabolized to BPAG by β-glucosylation. The other metabolites have not yet been identified; however, the chromatographic retention time of C was the same as that of synthetic BPA–di-β-glucoside. The accumulation of C was delayed compared with the rapid increase in concentration of BPAG after exposure of the cultured cells to [14C]BPA. This observation was consistent with the further metabolism of BPAG to C.

In the experiments with tobacco seedlings, the amount of radioactivity detected in the plants was only 30% of the radioactivity that disappeared from the medium at 0.5 h (Fig. 6A, B), suggesting that BPA might have been immediately but not
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Tightly bound to the surface of roots and released by washing-treatment. Incorporation of radioactivity in roots was slightly increased after 0.5 h but that in leaves was extensively increased during the incubation period (Fig. 6B). The increase of radioactivity in leaves was about two-thirds of the radioactivity that declined in the medium the during incubation period (Fig. 6A, B), suggesting that the rate of uptake of BPA by tobacco seedling is dependent on the ability to translocate and metabolize BPA from roots. The rate of BPA uptake by tobacco seedling calculated from Fig. 6A is $31 \mu g h^{-1} mg^{-1} FW$ of root. This value is equivalent to those of bacterial digestion (Sivavick et al. 1994).

Tobacco seedlings also took up BPA and metabolized it to the compounds that were observed in the cultured cells exposed to BPA (Fig. 7). Most of them were kept in the seedlings during the 4-h incubation period. Thus BPA is apparently metabolized in the same way in both cases by pathways which included a β-glucosylation step. Radioactivity incorporated into the compounds did not significantly increase in the roots, but increased in leaves during the 4-h labeling period, indicating that the radioactive compounds are translocated from roots to leaves. Among the metabolites, BPAG was the most abundant and markedly increased in leaves. These results indicate that BPAG may be a main metabolite of BPA to accumulate in the tobacco leaves.

While the total amount of radioactivity in the seedling increased during the incubation period, the amount of metabolites and BPA at 4 h was less than that at 2 h. This indicates that the seedlings processed BPA to other compounds that could not

Fig. 6  (A) Uptake of $[^{14}C]$BPA by tobacco seedlings. Filled circles, a root of 2-week-old tobacco seedling was immersed in 1.5 ml of distilled water with 8 kBq of $[^{14}C]$BPA, then incubated in the light; open circles, $[^{14}C]$BPA was supplemented to the medium without plants. The radioactivity of an aliquot (10 μl) of the medium was then determined by liquid scintillation counting at the indicated times. Vertical bars indicate standard deviations ($n = 3$). (B) Distribution of radioactivity in tobacco seedlings incubated in $[^{14}C]$BPA. A root of a 2-week-old tobacco seedling was immersed in 1.5 ml of distilled water containing 8 kBq of $[^{14}C]$BPA, then incubated for indicated times. At each sampling time, roots were washed with a large amount of water. Plants were divided into their roots, stems and leaves. These tissues were extracted with methanol. The radioactivity of an aliquot (100 μl) of the extract was determined by liquid scintillation counting. Vertical bars indicate standard deviations ($n = 3$).

Fig. 7  Levels of radioactive BPA and its metabolites in tobacco plants. Two-week-old tobacco seedlings were treated as described in Fig. 6. Methanol extracts of each tissue were analyzed by HPLC. (A) Roots; (B) leaves. Vertical bars indicate standard deviations ($n = 3$).
be detected under our HPLC conditions. With the methanol extract of 4-h incubated roots, about half of radioactivity was not eluted by 40% methanol and it was eluted by 2-propanol (data not shown), suggesting that highly hydrophobic compounds such as polyphenols might be produced from BPA or its metabolite during longer incubation.

Two systems have been reported to detoxify BPA: (1) the aerobic gram-negative bacterium MV1 degraded BPA to 4-hydroxybenzoic acid (Spivack et al. 1994); (2) BPA was conjugated with glucuronic acid to yield BPA-β-glucuronide in the mammalian liver prior to its excretion into the urine (Knaak and Sullivan 1966). The enzyme responsible for the conjugation, UDP-glucuronosyltransferase (EC 2.4.1.17), is the product of a multigene family and a specific isozyme for the glucuronosylation of BPA (UDP-glucuronosyltransferase 2B1) has been reported (Yokota et al. 1999). The work reported here has revealed tobacco plants can uptake and metabolize BPA by another metabolic pathway, β-glucosylation.

It has been well documented that plant cells have the ability to synthesize many phenolic compounds and some of these are conjugated by glucosylation and accumulate in the vacuole (Kreuz et al. 1996). The enzyme responsible for the glucosylation (UDP-glucose glucosyl transferase) is also the product of a multigene family and each isozyme has a specific substrate (Taguchi et al. 2001). Identification of the enzyme responsible for glucosylation of BPA will open the way for a study on the improvement of a plant’s ability to take up and detoxify BPA from water and soil.

**Materials and Methods**

**Cell cultures and BPA treatment**

Murashige-Skoog medium containing 16 μM of 2,4-D (MSD) was inoculated with tobacco BY-2 cells. The medium containing the cells in suspension was then incubated in the dark with gentle shaking for 2 weeks. A portion (30 ml) of the culture was then supplemented with BPA. Another portion (30 ml) was filtered and the collected cells were re-suspended in 30 ml of freshly prepared MSD. BPA was added to both the filtrate and the suspension of cells in freshly prepared MSD. For a control BPA was added to freshly prepared MSD. BPA was added to Murashige-Skoog medium containing 16 μM of 2,4-D (MSD). For a control BPA was added to freshly prepared MSD in the absence of cells. The initial concentration of BPA was 10 mg liter⁻¹ in all treatments, and cultures were incubated at 25°C with gentle shaking. Aliquots (10 μl) of the medium were taken and analyzed by HPLC (C-18 column 150×3.9 mm (Symmetry, Nihon Waters, Tokyo, Japan), 40°C, 40% aqueous methanol as eluent at a flow rate of 1 ml min⁻¹. Absorption at 217 nm was monitored.

**Analysis of the metabolites of BPA in BY-2 cells**

A portion (50 ml) of a 2-week-old suspension of BY-2 cultured cells was supplemented with 10 mg liter⁻¹ of BPA which served as a carrier for 410 kBq of propyl-2-[14]C]bisphenol A (Moravek Biochemicals, Brea, CA, U.S.A.) and incubated at 25°C with shaking. At each sampling time the radioactivity of an aliquot (25 μl) of the medium was determined by liquid scintillation counting (TRI-CARB; Packard Groningen, BK, Netherlands) and 20 μl of them was analyzed by HPLC. The radioactivity of each fraction (0.5 ml) was determined by liquid scintillation counting.

Ten milliliters of methanol was added to 3 ml of the culture and the mixture was allowed to stand for 24 h. The clear supernatant (50 μl) was then subjected to HPLC using the same conditions as above. The radioactivity of each fraction (0.5 ml) was determined by liquid scintillation counting.

**Isolation and identification of metabolite L**

A 2-week-old culture of 2 liters of BY-2 cells in suspension, supplemented with BPA at 10 mg ml⁻¹, was incubated at 25°C with gentle shaking for 3 h. The cells were then collected by filtration, washed with water, and extracted with 1 liter of methanol. The extract was evaporated until dry, dissolved in 200 ml of 67% aqueous methanol and the pH adjusted to 10 by the addition of a sodium hydroxide solution. Lipophilic material was removed by extracting three times with 200 ml of chloroform, and the aqueous phase was evaporated until dry after the pH was adjusted to 4 with acetic acid. The residue was extracted with 200 ml of chloroform at 50°C. The chloroform-soluble material was evaporated to dryness and dissolved in 2 ml of water. The aqueous solution was then subjected to HPLC (as above) with 20% aqueous acetonitrile as eluent. The fractions containing metabolite L were combined, concentrated and rechromatographed as before but with 35% aqueous methanol as an eluent. Again the fractions containing L were combined and were then evaporated to dryness to yield a chromatographically homogeneous product.

**Glycosidase digestion**

Aliquots (each containing a few micrograms) of metabolite L were incubated at 37°C for 2 h in the following enzyme/buffer solutions (50 μl): α-glucosidase (0.5 U), acetate (10 mM, pH 6.0), EDTA (1 mM); β-glucosidase (0.5 U), acetate (10 mM, pH 5.0); β-galactosidase (0.5 U), phosphate (10 mM, pH 7.5), MgCl₂ (10 mM), 2-mercaptoethanol (45 mM); α-mannosidase (6.5 U/M), citrate (4 M, pH 4.5); β-mannosidase (60 U/M), acetate (2 mM, pH 4.0). Each reaction product was then analyzed by HPLC using a C-18 column with 40% aqueous methanol as an eluent.

**Analysis of uptake and metabolism of BPA by tobacco plants**

Seedlings of tobacco (Nicotiana tabacum L. cv. Xanthii NC) were grown on soil in a glasshouse for 2 weeks at 25°C during the day and 20°C at night with a constant relative humidity of 70%. The seedlings were then transferred to an environment-controlled chamber. Their roots were washed with distilled water and a root of each seedling was immersed in 1.5 ml of 10 mg liter⁻¹ BPA solution containing 8 kBq of [14]C]BPA, then incubated at 20°C under fluorescent lamps (100 μmol m⁻² s⁻¹ of photosynthetically active photon flux density). At each sampling time, roots were washed with large amount of water. Then plants were divided into their roots, stems and leaves. These tissues were extracted with a few milliliters of methanol, and extracts were evaporated to dryness and then dissolved with 0.1–0.2 ml of 40% methanol. Aliquot of the extracts was analyzed by HPLC as described above.

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References


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