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# CYTOTOXIC EFFECTS OF BIPHENYL AND HYDROXYBIPHENYLS ON ISOLATED RAT HEPATOCYTES

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**Abstract**—The cytotoxic effects of biphenyl (BP) and its hydroxylated derivatives, *o*-phenylphenol (OPP), *m*-phenylphenol (MPP), *p*-phenylphenol (PPP), 2-biphenylyl glycidyl ether (OPP-epoxide), phenyl-hydroquinone (PHQ), *o,o'*-biphenol (*o,o'*-BPol) and *p,p'*-biphenol (*p,p'*-BPol), were investigated in freshly isolated rat hepatocytes. OPP, MPP and PPP, at concentration of 0.75 mM, resulted in the loss of intracellular ATP, glutathione (GSH) and protein thiols, causing cell death. OPP-epoxide and BP were less toxic than the OPP isomers. MPP or PPP compared with OPP caused serious impairments in oxidative phosphorylation in mitochondria isolated from rat liver. PHQ (0.75 mM) caused a rapid loss of intracellular ATP which preceded the onset of cell death. PHQ was more toxic than *o,o'*-BPol or *p,p'*-BPol. PHQ dissolved in Krebs–Henseleit buffer without hepatocytes was rapidly converted to its corresponding quinone, phenyl-benzoquinone. The cytotoxicity produced by PHQ depends on the rate of formation of reactive intermediates. These results indicate that the addition of a hydroxyl group to the aromatic ring of BP enhances BP-induced cytotoxicity and that the mitochondria are a common target of the OPP isomers and other BP derivatives. In addition, the *para-* or *meta-*hydroxyl groups rather than the *ortho-*hydroxyl group increase the toxicity. The cytotoxicity produced by PHQ depends on the rate of formation of reactive intermediate(s) such as phenyl-benzoquinone.

Biphenyl (BP‖) and *o*-phenylphenol (OPP, 2-hydroxybiphenyl) are broad spectrum antimicrobials and are utilized as fungicides and anti-bacterial agents in the post-harvest treatment of fruits and vegetables. Due to their widespread use, both compounds have been investigated *in vivo* and *in vitro* to assess various toxicological properties. Acute and chronic effects [1, 2], cytogenetic effects [3, 4], mutagenicity [5, 6], teratogenicity [7, 8] and immunological effects [9] have been demonstrated after exposure to both compounds. Liver [10, 11], kidney [1, 10, 12] and urinary bladder [13] have been suggested as important target organs in rats treated with a single large dose or with chronic low doses of each compound. In previous studies on OPP and its metabolites, we have demonstrated that at least two mechanisms are involved in OPP-induced cytotoxicity [14, 15]. The first is the disturbance of mitochondrial respiration by the direct action of the parent compound. The second is through interactions between intermediates derived from OPP and mitochondrial and other cellular functions. The metabolism of BP *in vivo* [16–18] and *in vitro* [19–21] is well established; BP is metabolized by the microsomal monooxygenase system, predominantly to *p*-phenylphenol (PPP, 4-hydroxybiphenyl) and to a lesser extent to OPP and *m*-phenylphenol (MPP, 3-hydroxybiphenyl). Some of these hydroxylated biphenyls are then conjugated to glucuronide or sulfate in intact liver cells. Despite the metabolic details known about BP and its hydroxylated derivatives in rats, no extensive studies have been performed on the relationship between these compounds and their toxicities in hepatocytes. Here, using freshly isolated rat hepatocytes, we report on the comparative toxic effects of BP and its hydroxylated biphenyls on freshly isolated rat hepatocytes. In addition,we have investigated the effects of the location of hydroxyl groups on cytotoxicity.

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‖ Abbreviations: BP, biphenyl; OPP, *o*-phenylphenol; MPP, *m*-phenylphenol; PPP, *p*-phenylphenol; OPP-epoxide, 2-biphenylyl glycidyl ether; PHQ, phenyl-hydroquinone; *o,o'*-BPol, *o,o'*-biphenol; *p,p'*-BPol, *p,p'*-biphenol; GSH, glutathione; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*-(2-ethanesulfonic acid); DMSO, dimethyl sulfoxide; RCI, respiratory control index; PBQ, phenyl-benzoquinone.

## MATERIALS AND METHODS

*Materials.* Chemicals were purchased from the following companies: BP, OPP, PPP, *o,o'*-biphenol (*o,o'*-BPol) and *p,p'*-biphenol (*p,p'*-BPol) (purities >98%) from the Tokyo Kasei Co. (Tokyo, Japan); MPP (purity >90%) and 2-biphenylyl glycidyl ether (OPP-epoxide, purity >95%) from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.); reduced glutathione (GSH) and oxidized forms, and bovine serum albumin from the Sigma Chemical Co. (St Louis, MO, U.S.A.); and collagenase from the Wako Pure Chemical Ind. (Osaka, Japan). All other chemicals were of the highest purity commercially

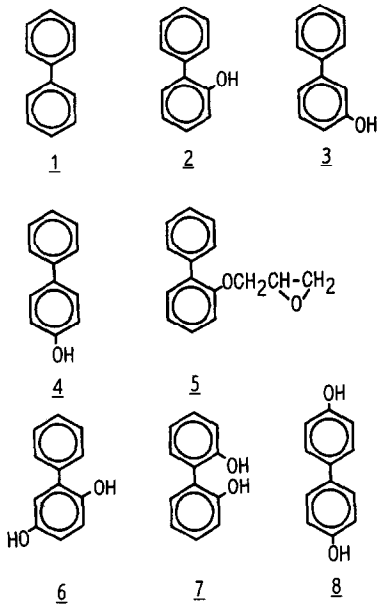


Fig. 1. Chemical structures of BP and its hydroxylated derivatives utilized in this study. (1) BP, (2) OPP, (3) MPP, (4) PPP, (5) OPP-epoxide, (6) PHQ, (7) *o,o'*-BPol, (8) *p,p'*-BPol.

available. Chemical structures of BP and of its hydroxylated derivatives used are shown in Fig. 1.

*Isolation and incubation of hepatocytes.* Male Fischer-344 rats (240–280 g) were used in all experiments. Hepatocytes were isolated by collagenase perfusion of liver as described by Moldéus *et al.* [22]. Viability of hepatocytes was assessed by counting the percentage of hepatocytes which excluded Trypan blue. Initial cell viabilities were approximately 90%. Hepatocytes ($10^6$ cells/mL) were suspended in Krebs–Henseleit buffer (pH 7.4) containing 12.5 mM Hepes and 0.1% albumin. All incubations were performed in rotating, round-bottomed flasks at 37° under a constant flow of humidified carbogen (95% $O_2$ and 5% $CO_2$). Reactions were initiated by the addition of BP or its derivatives dissolved in dimethyl sulfoxide (DMSO) (final concentration less than 1%). The corresponding control groups were added to an equivalent volume of DMSO. Aliquots of cell suspensions were taken at intervals for the determination of cell death as well as for quantification of the concentrations of GSH, ATP, protein thiols and protein.

*Preparation of liver mitochondria.* Liver mitochondria were isolated from male Fischer-344 rats by differential centrifugation in medium containing 0.25 M sucrose, 5 mM Tris–HCl (pH 7.4) and 1 mM EDTA for measurement of respiration rates [23]. EDTA was omitted in the final wash and resuspension.

*Measurement of respiration rates.* The rate of oxygen consumption was measured polarographically with a Clark-type oxygen electrode (Yellow Springs Instruments Co., Model 5300) at 25° in the presence (state 3) and exhaustion (state 4) of 0.1 mM ADP [23]. Respiration buffer (3 mL, pH 7.4) contained 0.2 M sucrose, 20 mM KCl, 3 mM $MgCl_2$, 5 mM

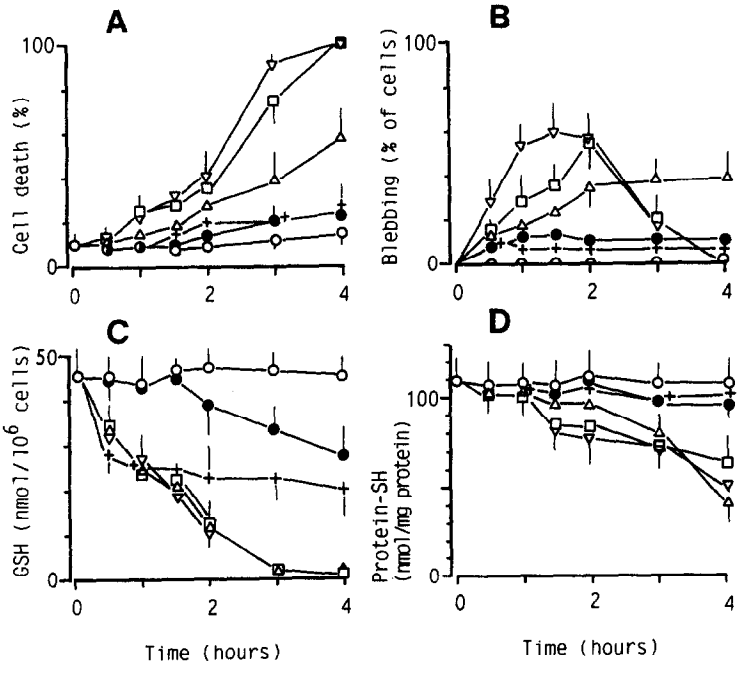


Fig. 2. Effects of BP and hydroxybiphenyls on cell viability (A), cell blebbing (B) and levels of GSH (C) and protein thiols (D) of isolated rat hepatocytes. Hepatocytes were incubated at $10^6$ cells/mL in Krebs–Henseleit buffer, pH 7.4, with no addition (○), 0.75 mM of BP (●), OPP (△), MPP (□), PPP (▽) and OPP-epoxide (+) as described in Materials and Methods. Results are expressed as the mean ± SE of three separate experiments.

potassium phosphate and 1 μM rotenone. The respiration substrate was 5 mM succinate and the amount of mitochondria was 1 mg protein/mL. The respiratory control index (RCI) was calculated as the ratio of state 3/state 4 respiration.

*Biochemical assays.* Adenine nucleotides in hepatocytes were measured using HPLC according to the procedure of Jones [24].

Cellular GSH levels were determined by HPLC essentially as described by Reed *et al.* [25].

Reduced protein thiol concentrations were determined by using Ellman's reagent as described previously [26].

Protein was determined by the method of Lowry *et al.* [27] using bovine serum albumin as a standard.

Blebbing of hepatocytes was assayed by light microscopy and expressed as the percentage of Trypan blue excluding cells which exhibited multiple surface protrusions [28].

## RESULTS

The concentration of compounds used (0.75 mM) was based on previous experiments of OPP-induced cytotoxicity; 0.75 mM OPP was moderately toxic and resulted in approximately 50% cell death within 3 hr [14]. Although the addition of BP or OPP-epoxide to the hepatocyte suspension was not markedly cytotoxic during the incubation period, OPP and its isomers, MPP and PPP, caused a time-dependent cell death accompanied by depletion of cellular GSH and protein thiols (Fig. 2). The rapid loss of cellular GSH induced by OPP isomers was followed by the depletion of protein thiols. The appearance of surface blebs preceded the onset of cell death induced by OPP and its isomers. The frequency of cell blebbing was correlated with the cytotoxicity. The onset of surface blebs induced by PPP was faster than that induced by MPP or OPP.

Figure 3 shows the effects of BP, OPP isomers and OPP-epoxide on the levels of adenine nucleotides in hepatocytes. The abrupt depletion of cellular ATP caused by OPP and its isomers, especially PPP, was reflected in a concomitant temporary increase in levels of ADP and AMP. Total amount of nucleotides pool in hepatocytes treated with OPP isomers was gradually depleted with the incubation period. The rapid loss of ATP and induction of cell blebbing caused by PPP or MPP preceded the onset of cell death (Figs 2 and 3). Based on the rate of cell death and loss of ATP, PPP is the most toxic, followed by MPP and OPP.

Inhibition of oxidative phosphorylation is one mechanism by which OPP or its metabolites (phenylhydroquinone, PHQ and phenyl-benzoquinone, PBQ) can cause depletion of intracellular ATP levels. The effects of BP, OPP isomers and OPP-epoxide on the oxygen consumption by isolated mitochondria is shown in Table 1. Addition of 100 or 250 μM of OPP isomers resulted in a concentration-dependent increase in the rate of state 4 oxygen consumption indicating partial uncoupling of mitochondria. The effect of MPP or PPP was greater than that of OPP. In contrast, state 3 oxygen consumption was inhibited with these isomers and the potency was OPP > MPP, PPP > BP, OPP-epoxide. Therefore, inhibition of RCI, a sensitive index of mitochondrial impairment, was due both to an inhibition of state 3 respiration and to a stimulation of state 4 respiration.

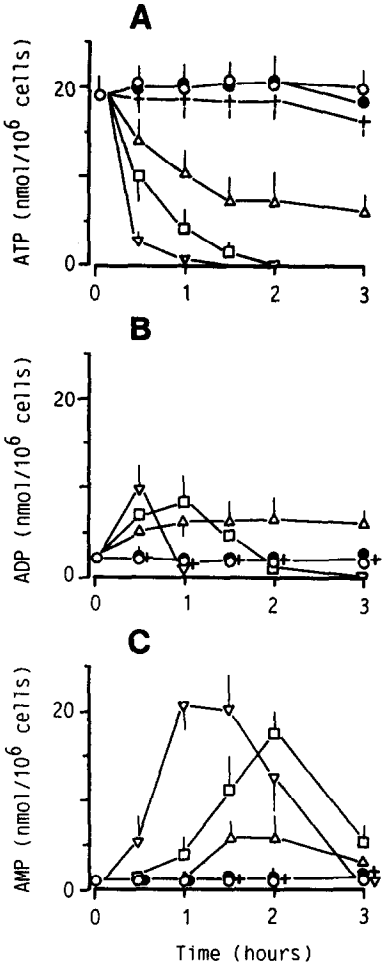


Fig. 3. Effects of BP and its hydroxylated derivatives on levels of ATP (A), ADP (B) and AMP (C) of isolated rat hepatocytes. Hepatocytes were incubated at $10^6$ cells/mL in Krebs–Henseleit buffer, pH 7.4, with no addition (○), 0.75 mM of BP (●), OPP (△), MPP (□), PPP (▽) and OPP-epoxide (+). Results are expressed as the mean ± SE of three separate experiments.

The cytotoxic effects of PHQ, *o,o'*-BPol and *p,p'*-BPol on isolated rat hepatocytes were investigated (Fig. 4). The addition of PHQ (0.75 mM) caused cell death, accompanied by rapid depletion of intracellular ATP and levels of GSH and protein thiols. *o,o'*-BPol or *p,p'*-BPol did not significantly affect cell viability during a 3 hr incubation period. The cell death caused by PHQ was accompanied by rapid depletion of intracellular ATP and levels of GSH and protein thiols. The loss of cellular ATP level induced by PHQ or *o,o'*-BPol was reflected in a concomitant increase in the levels of ADP and AMP 60 min later (Table 2). As shown in Table 3, PHQ and *p,p'*-BPol caused a decrease in the rate of state 3 oxygen consumption by isolated mitochondria, whereas *o,o'*-BPol decreased rates in both state 3

Table 1. Effects of BP and hydroxybiphenyls on mitochondrial respiration

| Treatment | (μM) | Mitochondrial respiration (ng atom O/mg protein/min) State 4 | State 3 | RCI |
|---|---|---|---|---|
| None | | 15.3 ± 2.2 | 57.7 ± 1.5 | 3.77 |
| BP | 100 | 14.7 ± 1.9 | 53.6 ± 2.6 | 3.64 |
| | 250 | 15.3 ± 3.4 | 48.9 ± 1.7 | 3.19 |
| OPP | 100 | 15.7 ± 1.7 | 39.1 ± 1.6 | 2.49 |
| | 250 | 19.8 ± 1.0 | 24.3 ± 0.7 | 1.22 |
| MPP | 100 | 22.9 ± 1.7 | 44.1 ± 3.7 | 1.92 |
| | 250 | 35.4 ± 2.9 | 38.2 ± 2.1 | 1.08 |
| PPP | 100 | 23.2 ± 1.9 | 46.3 ± 1.4 | 1.99 |
| | 250 | 37.9 ± 4.2 | 37.3 ± 3.2 | 0.98 |
| OPP-epoxide | 100 | 14.0 ± 1.4 | 51.2 ± 1.6 | 3.65 |
| | 250 | 18.6 ± 1.0 | 50.9 ± 2.7 | 2.73 |

Mitochondria (1 mg/mL) were preincubated in 3 mL of respiration buffer, containing succinate (5 mM) and rotenone (1 μM), for 1.5 min at 25° (see Materials and Methods). For the measurement of state 3 respiration, BP or its derivatives were incubated with mitochondria for 1.5 min before the addition of ADP (100 μM). RCI was calculated as the ratio of state 3/state 4 respiration. Values are the mean ± SE of three determinations.

Table 2. Effects of dihydroxybiphenyls on the level of adenine nucleotides in isolated hepatocytes

| Treatment | Adenine nucleotides (nmol/$10^6$ cells) ATP | ADP | AMP | Total |
|---|---|---|---|---|
| None | 17.1 | 3.6 | 0.2 | 20.9 |
| PHQ | 5.8 | 7.9 | 8.1 | 21.8 |
| *o,o'*-BPol | 9.9 | 6.2 | 3.6 | 19.7 |
| *p,p'*-BPol | 16.9 | 3.9 | 0.3 | 21.1 |

Hepatocytes were incubated with dihydroxybiphenyls (0.75 mM), PHQ, *o,o'*-BPol and *p,p'*-BPol, for 60 min at 37° as described in Materials and Methods.

Values are the means of two separate experiments.

and 4 oxygen consumption. According to RCI, it follows that the order of impairment potency is *o,o'*-BPol > *p,p'*-BPol, PHQ.

As some hydroquinones are converted to the corresponding quinones via semiquinones by autoxidation, structural change in these dihydroxybiphenyls dissolved in Krebs–Henseleit buffer without cells were monitored by their absorption spectra (240–420 nm) changes (Fig. 5). Although PHQ was converted to PBQ (a characteristic absorption peak, 373.7 nm) with time, no significant spectral change in either *o,o'*-BPol or *p,p'*-BPol was found during the 60 min incubation period. Further, PBQ formation and/or PHQ loss were accompanied by oxygen consumption in the buffer (data not shown). These results suggest that the cytotoxicity caused by PHQ is associated with the formation of the quinone, PBQ.

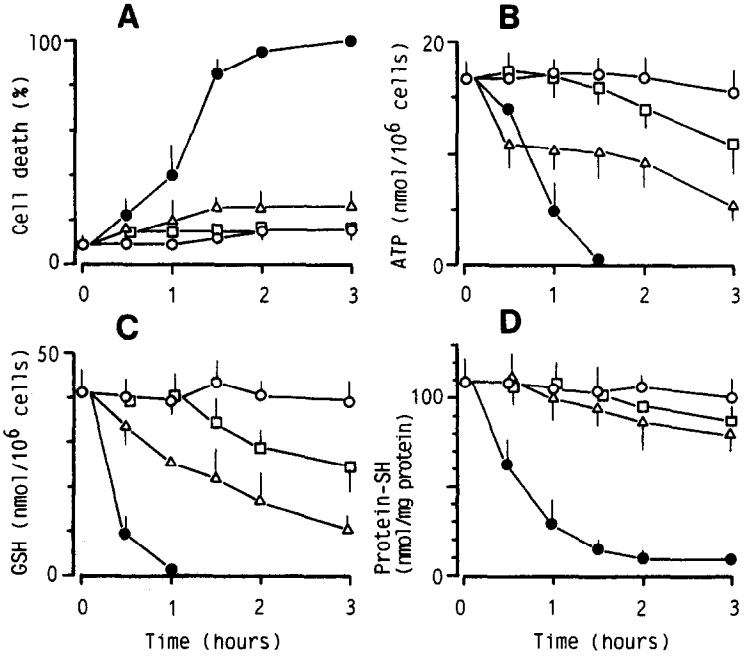


Fig. 4. Effects of dihydroxybiphenyls on cell viability (A), and levels of ATP (B), GSH (C) and protein thiols (D) of isolated rat hepatocytes. Hepatocytes were incubated at $10^6$ cells/mL in Krebs–Henseleit buffer with no addition (○), 0.75 mM of PHQ (●), *o,o'*-BPol (△) and *p,p'*-BPol (□). Results are expressed as the mean ± SE of three separate experiments.

Table 3. Effects of dihydroxybiphenyls on mitochondrial respiration

| Treatment | (μM) | Mitochondrial respiration (ng atom O/mg protein/min) State 4 | State 3 | RCI |
|---|---|---|---|---|
| None | | 16.5 ± 2.3 | 77.0 ± 4.0 | 4.66 |
| PHQ | 250 | 13.9 ± 0.9 | 60.2 ± 3.3 | 4.33 |
| | 500 | 14.6 ± 1.7 | 51.1 ± 2.5 | 3.51 |
| *o,o'*-BPol | 250 | 8.72 ± 1.1 | 8.77 ± 1.6 | 1.01 |
| | 500 | 5.67 ± 1.9 | 5.45 ± 2.2 | 0.96 |
| *p,p'*-BPol | 250 | 18.9 ± 2.7 | 63.8 ± 6.1 | 3.38 |
| | 500 | 21.8 ± 0.8 | 57.7 ± 4.4 | 2.64 |

Mitochondria (1 mg protein/mL) were preincubated in 3 mL of respiration buffer, containing succinate (5 mM) and rotenone (1 μM), for 1.5 min at 25° (see Materials and Methods). For measurement of state 3 respiration, each dihydroxybiphenyl was incubated with mitochondria for 1.5 min before the addition of ADP (100 μM). RCI was calculated as the ratio of state 3/state 4 respiration.

Values are the mean ± SE of three determinations.

## DISCUSSION

The present results indicate that OPP and its isomers, MPP and PPP, are cytotoxic to isolated rat hepatocytes and that they are more toxic than BP or OPP-epoxide, which is substituted by glycidyl ether at the hydroxyl group of OPP (Fig. 2). These OPP isomers depleted intracellular ATP and GSH, which consistently preceded cell death (Figs 2 and 3). PPP and MPP especially caused impairments of mitochondrial function related to oxidative phosphorylation (Table 1). Based on these results, the order of toxic potency is PPP, MPP > OPP > BP, OPP-epoxide. Since the introduction of a hydroxyl group to the aromatic ring leads to an increase in toxicity, it seems likely that the substituted hydroxyl group plays an important role in the induction of cytotoxicity.

Mitochondria are the main source of energy production in hepatocytes. Several studies have reported that a decline in cellular ATP levels is critical in the development of cell damage [15, 29, 30]. In fact, an abrupt loss of ATP level, with a concomitant increase in intracellular ADP and AMP, consistently preceded cell death (Figs 2 and 3). As OPP and its isomers do not react with ATP in Krebs–Henseleit buffer without hepatocytes (data not shown), the depletion of intracellular ATP may be due to the inhibition of the adenine nucleotide synthesis system and/or the activation of hydrolysis of ATP by these isomers. Since the maintenance of ATP levels is important for polymerization of microfilaments and microtubules, its depletion might lead to cytoskeletal disruption [31, 32]. Orrenius *et al.* [33] have proposed that some chemically induced cytotoxicities are associated with increased cytosolic $Ca^{2+}$ concentrations accompanied by the formation of blebbing of the cell surface. We have shown that OPP and its metabolites, PHQ and PBQ, cause the release of $Ca^{2+}$ from isolated rat mitochondria [14]. Thus, the cell blebbing may result from the perturbation of intracellular ATP and/or $Ca^{2+}$ homeostasis. Consequently, it would appear that ATP depletion is not the result of cell death but rather may be the cause.

The loss of intracellular ATP was associated with

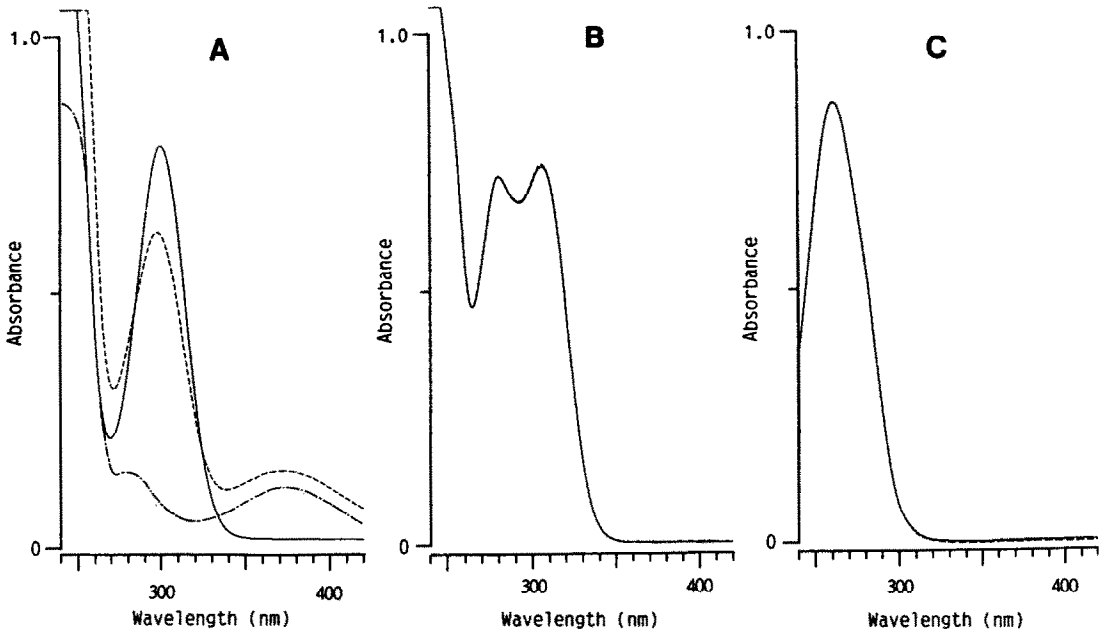


Fig. 5. Changes in absorption spectra of PHQ (A), *o,o'*-BPol (B) and *p,p'*-BPol (C). These spectra of 0.15 mM PHQ, 0.15 mM *o,o'*-BPol and 0.05 mM *p,p'*-BPol dissolved in Krebs–Henseleit buffer (pH 7.4) at 30° were monitored at 0 min (——) and 60 min (- - - -). Characteristic absorption peaks of PHQ and PBQ (— · —, in A) were at 300.1 and 373.7 nm, respectively.

the impairment of mitochondrial respiration (Fig. 3 and Table 1). ATP breakdown to ADP occurs rapidly in cells exposed to hypoxia or to inhibitors of respiration [29, 34]. State 3 respiration was inhibited by BP and its monohydroxylated biphenyls. This inhibition is generally considered to reflect an interference with electron transport. Since phenols are effective inhibitors of a number of FAD- and $NAD^+$-containing oxidases and dehydrogenases via reaction mechanisms that exhibit complex kinetics [35, 36], this suggests that the electron transport chain in mitochondria is affected by BP and its hydroxylated derivatives. In addition, the increase in state 4 respiration caused by OPP, MPP and PPP indicates uncoupling of oxidative phosphorylation in mitochondrial respiration. The impairment potency assessed by RCI is PPP, MPP > BP, OPP > OPP-epoxide.

It is well known that the sulfhydryl group in proteins and non-proteins is involved in the maintenance of various cellular functions. Some investigators have indicated that protein thiols, more than non-protein thiols, are critical for the maintenance of cell viability during toxic chemical insults [37–39]. The loss of cellular GSH may be partly due to the depletion of ATP, which is required for GSH synthesis [40]. In this study, we show a time lag for the onset of protein thiol loss (Fig. 2). At extremely low concentrations of intracellular GSH, cell viability when exposed to xenobiotics correlates with the maintenance of protein thiol levels [41]. Although the mechanism that ultimately leads to rapid irreversible loss of intracellular GSH and to gradual loss of protein thiols caused by OPP isomers is not clear, the depletion of protein thiols by the compounds may result in a disturbance for cell viability as an irreversible effect.

BP is metabolized by the microsomal mixed-function oxygenase, predominantly to PPP with smaller amounts of OPP and MPP [19]. Therefore, BP-induced toxicity may result from intermediate PPP as well as the direct action of parent compound. Subsequently, OPP and PPP are converted to PHQ and *p,p'*-BPol by the microsomal monooxygenase system, respectively [19]. PHQ then converts to the reactive intermediate PBQ via a PHQ semiquinone radical by autoxidation. PBQ then reacts rapidly with intracellular protein thiols and GSH [15, 42–44]. The cytotoxicity of PHQ is dependent on the formation and accumulation of PBQ and on the generation of a reactive oxygen species which does not directly affect the induction of cytotoxicity of PHQ [37]. However, *o,o'*-BPol and *p,p'*-BPol are less toxic than PHQ (Fig. 4 and Table 2). Although the addition of hydroxyl groups to the aromatic ring of BP leads to a decrease in hydrophobicity of the molecule, this does not correlate with the cytotoxicity of *o,o'*-Biol or *p,p'*-BPol. This indicates that the position of the hydroxyl group added to BP is important to the induction of cytotoxicity. Both *o,o'*-BPol and *p,p'*-BPol are more stable than PHQ in Krebs–Henseleit buffer without hepatocytes (Fig. 5). These results suggest that the cytotoxicity caused by PHQ is attributable to one-electron oxidation, to give the corresponding quinone via the semiquinone radical. Taken together with the present results, the acute cytotoxicity caused by hydroxybiphenyls involved numerous mechanisms which cause impairment of mitochondrial respiration and loss of ATP, GSH and protein thiols, and may involve irreversible binding of active intermediates to macromolecules and perturbations in intracellular $Ca^{2+}$ homeostasis.

In conclusion, hydroxybiphenyls cause a number of interrelated biochemical effects which together result in loss of ATP followed by cell death. The cytotoxicity caused by BP is increased by the addition of a hydroxyl group. *P*- and *m*-hydroxyl groups rather than *o*-hydroxyl group tend to increase toxicity. The respiration system of mitochondria is a common target for OPP isomers and BP derivatives.

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