Table.1 Estrogen-like Activities of Phenols

Compound	Concentration Induced Maximum Activity (M)	Induced Units	% of 17β-Estradiol (10^{-8}M)
Phenol	$>10^{-3}$	—	—
4-*tert*-Butylphenol	10^{-4}	95	17.3
4-*sec*-Butylphenol	3×10^{-4}	374	68.2
3-*tert*-Butylphenol	$>10^{-3}$	—	—
2-*tert*-Butylphenol	$>10^{-3}$	—	—
2-*sec*-Butylphenol	$>10^{-3}$	—	—
4-*tert*-Pentylphenol	10^{-5}	126	23.0
4-*tert*-Octylphenol	10^{-5}	170	31.0
4-*n*-Octylphenol	$>10^{-3}$	—	—
4-*n*-Nonylphenol	$>10^{-3}$	—	—
Bisphenol A	10^{-5}	164	29.9
17β-Estradiol	(10^{-9})	548	100

ロゲン様活性の最大活性を示す暴露濃度が異なっていた．これらのことは，パラ位に結合するアルキル基は構造的に一定以上の空間を占めることが必要であることを示唆している．ビスフェノールAの場合にも，この推論が適合している．被験化学物質のエストロゲン様活性を，10^{-8} Mの17β-エストロゲンを暴露した際のエストロゲン活性に換算すると，最大の活性を示した4-*sec*-ブチルフェノールでは30000倍の濃度で68％相当の活性であった．また，4-*tert*-ブチルフェノールでは10000倍の濃度で，17％相当の活性を示した．4-*tert*-オクチルフェノール，4-*tert*-ペンチルフェノールおよびビスフェノールAでは1000倍の濃度でそれぞれ31％，23％および30％相当の活性であった．側鎖が*tert*構造を持つこれらの化学物質の間では，結合・解離を総括した親和性として10倍の差が認められるながら，エストロゲン様活性には大きな差が認められない結果であった．これだけの結果からは結論を導き出すのは難しいが，側鎖の*tert*構造と空間的に占める体積が，エストロゲンα受容体のリガンド結合領域がつくる空間と原子間の配位との関係で重要な要素であると推測される．また，側鎖の*sec*構造はエストロゲンα受容体との親和性については低いが，解離の程度が悪くエストロゲン様活性を誘導する能力を長期に維持する結果，エストロゲン様活性が高く出たのではないかと考えられる．

以上の結果から，エストロゲン様活性を示す化学物質は，酵母Two-Hybrid Systemで解析するかぎりでは，①フェノール骨格を持ち，②水酸基に対してパラ位に，③－C－3Cがとる立体空間を持ち，④分岐した炭素鎖を持つ構造を持つと考えられる．エストロゲン様活性が未知の化学物質について，エストロゲン様活性の有無を推測するには上記の要件は一つの指標となりうるであろう．ここでは生化学的な結果のみに基づく推論であるため，コンピュータなどによるエストロゲン受容体と化学物質の立体構造相互作用シミュレーション解析を進めることによりさらに明確な結論が導かれるものと期待される．

また，ここで仮説を導く根拠とした試験結果は酵母細胞の試験系を用いており，対象とする化学物質の細胞膜透過性については考慮がなされていない．対象とした化学物質が膜を透過しないとエストロゲン受容体と結合できないことから，化学物質の膜透過性の程度が結果に影響していることも考慮しておかなければならない．また，ヒトを対象とする場合，酵母と哺乳動物細胞との膜の違いがあり，化学物質の構造上透過性に差違があることも考えられ，哺乳動物の内分泌かく乱性を考えた場合，注意しておく必要があるであろう．

2. オクチルフェノールエトキシレート類およびノニルフェノールエトキシレート類のエストロゲン様活性

界面活性剤などの原料として使用されているオクチルフェノールポリエトキシレートやノニルフェノールポリエトキシレートは，環境中に排出された後，微生物などにより生分解されてジエトキシレートやモノエトキシレート体になることが知られている．そこで，4-*tert*-オクチルフェノールエトキシレート，4-*tert*-オクチルフェノールジエトキシレート，ノニルフェノールエトキシレートおよびノニルフェノールジエトキシレートのエストロゲン様活性を酵母Two-Hybrid Systemにより測定したが，有意な活性は検出されなかった．他の検出方法ではエストロゲン様活性が認められることから，酵母の系ではこれらの化学物質の膜透過性が低く細胞内に取り込まれずエストロゲン受容体と結合できなかったのではないかと推測された．

3. ビフェニル類のエストロゲン様活性

ビフェニル，*o*-ヒドロキシビフェニル，*m*-ヒドロキシビフェニル，*p*-ヒドロキシビフェニル，2,2'-ジヒドロキシビフェニルおよび4,4'-ジヒドロキシビフェニルについてエストロゲン様活性を酵母Two-Hybrid Systemにより測定した（Fig.2）．ビフェニルおよび2,2'-ジヒドロキシビフェニルはエストロゲン様活性が認められなかったが，*p*-ヒドロキシビフェニルは10^{-6} Mから3×10^{-4} Mの濃度範囲で活性が見られ，3×10^{-5} Mの濃度で最高活性を示した．17β-エストラジオールの10^{-8} Mの活性に対して35.9％相当であった．*o*-ヒドロキシビフェニルおよび*m*-ヒドロキシビフェニルでは10^{-3} Mから10^{-5} Mの濃度範囲で，最高活性として10^{-4} Mの濃度において極わずかであるが検出された．4,4'-ジヒドロキシビフェニルでは3×10^{-5} Mから10^{-3} Mの濃度範囲で，3×10^{-4} Mの濃度で17β-エストラジオールの10^{-8} Mの活性に対して55.9％相当の最高の活性を示した．水酸基に対して

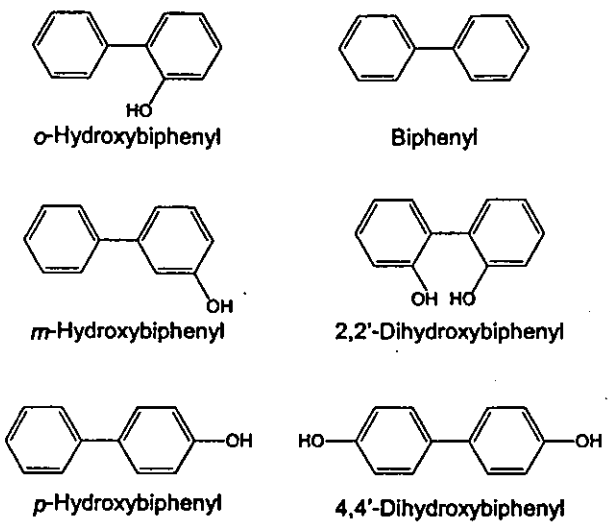

Fig.2　Structures of biphenyl derivatives

パラ位にベンゼン環がついたp-ヒドロキシビフェニルおよび4,4'-ジヒドロキシビフェニルで強いエストロゲン様活性が認められた結果は，先に推論した仮説を支持するものであった．

さらに，これらのビフェニル類をラット肝ミクロソーム画分により代謝を行い，エストロゲン様活性を測定した．最終濃度として10％S9，4 mM $MgCl_2$，16.5 mM KCL，2.5 mM Glucose-6-phosphate，2 mM NADPH，2 mMNADH，50 mM Na-PO_4（pH 7.4）の組成をもつ溶液を調整してエストロゲン様活性測定反応液に添加し，エストロゲン活性の反応時に代謝を同時に行った．S9は，5,6-ベンゾフランとフェノバルビタールを腹腔内投与し，酵素誘導をかけたラットの肝臓から調製したミクロソーム画分である．代謝することによりエストロゲン様活性が有意に増加する傾向が認められた（Table.2）．ビフェニルは代謝を行わない場合は活性が検出されなかったのに対し，代謝を行うと10^{-8} Mの濃度の17β-エストラジオールを暴露した際の活性に換算すると33％相当の活性が見られるようになった．同様に，m-ヒドロキシビフェニル，p-ヒドロキシビフェニルおよび4,4'-ジヒドロキシビフェニルではそれぞれ，2.4％が24.4％，35.9％が75.8％，55.9％が107.1％相当の活性の増加が認められた．ここで行った代謝は主として酸化反応を促進するものであり，結果的に水酸基が付加する反応であることを考えると，代謝反応をおこなうことにより水酸基が付加したフェノール構造が生じて，エストロゲン様活性の増加が見られたと考えられる．代謝反応後の生成物の構造を同定していないため，結論を述べることは出来ないが，上記の仮説を支持する結果である．

4．おわりに

エストロゲンα受容体に結合親和性のある化学物質は，酵母Two-Hybrid Systemによる活性から推測するところでは，①フェノール骨格を持ち，②水酸基に対してパラ位に，③－C－3Cがとる立体空間を持ち，④分岐した炭素鎖を持つ構造であるとの推論を得た．したがって，このような構造を持つ化学物質は内分泌かく乱性を有する候補物質と推測される．

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(2) 2段階形質転換試験のプロモーション期に認められる発がんプロモーターによるNP95 mRNAの過剰発現

衛生微生物部　酒井綾子，高鳥浩介

［目的］

本研究は，発がんプロモーターが誘導する多様な遺伝子発現変化を分析し，化学物質による発がん促進の機序を解析するとともに，遺伝子発現変化を指標とする簡便な発がんプロモーター検出法を開発することを目的として行っている．BALB/3T3細胞2段階形質転換実験系を2段階発がんのin vitroモデル系として用いている．第一次で，作用機序の異なる3つの発がんプロモーター，12-O-テトラデカノイルホルボール-13-アセテート

Table.2　Estrogen-like Activities of Biphenyls

Compounds	Excretion rate [% of total]						
	In urine				In feces		
	0-5.5 hr	5.5-24.5 hr	24.5-54 hr	0-54 hr	0-24.5 hr	24.5-54 hr	0-54 hr
TA					49.48±1.47	13.26±1.88	62.74±2.36
GA	0.00±0.00[1)]	0.01±0.00	ND[2)]	0.01±0.00	0.16±0.02	0.03±0.01	0.19±0.02
4-OMGA	0.02±0.00	0.07±0.01	ND	0.10±0.01	ND	ND	ND
PY	0.00±0.00	0.24±0.13	ND	0.24±0.13	0.02±0.01	ND	0.02±0.01
RE	0.00±0.00	1.87±0.65	0.18±0.05	2.06±0.70	0.65±0.04	0.10±0.01	0.76±0.04

Abbreviations are: TA, tannic acid; GA, gallic acid; 4-OMGA, 4-O-methyl gallic acid; PY, pyrogallol; and RE, resorcinol.
1) Data are means ±SEM for 6 rats.
2) ND, not detected.

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Bisphenol A induces apoptosis in central neural cells during early development of *Xenopus laevis*☆

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Abstract

Bisphenol A (BPA), known to be a xenoestrogen, is widely used in industry and dentistry. In the present study, we investigated the effects of BPA on the early development of *Xenopus laevis* embryos. Stage 6 embryos were exposed to 10–100 μM BPA. Developmental abnormalities were observed when the embryos were exposed to at least 20 μM BPA, with marked developmental abnormalities, such as crooked vertebrae and developmental defects of the head and abdomen, detected in all embryos up to stage 40. Interestingly, apoptosis occurred specifically in central nervous tissue cells of the brain and spinal cord, as assessed by histological analysis. BPA-induced malformations and apoptosis were not observed in embryos exposed to BPA after stage 10. When embryos were exposed to 10 μM 17β-estradiol (E2), abnormalities were also observed until stage 40. However, the abnormalities induced by BPA and E2 were different and E2 exposure did not induce apoptosis in the central nervous system. Our results indicated that the developmental abnormalities and apoptosis induced by BPA exposure were not inhibited by the addition of E2. In conclusion, we demonstrated that BPA induced marked malformations and specific apoptosis of central nervous system cells during early development of *X. laevis* embryos, and that these BPA effects appeared to be due to non-estrogenic activities on developmental processes.

Keywords: *Xenopus laevis*; Amphibian; Embryo; Early development; Bisphenol A; 17β-Estradiol; Endocrine disruptors; Xenoestrogen; Estrogen receptor; Malformation; Apoptosis; Brain; TUNEL; Histological analysis; Exposure test; Central nerve cell

It has been shown that many chemicals in the environment can disrupt endocrine functions and cause adverse effects on the reproductive functions of humans and animals [2]. Although xenoestrogens share no structural homology with estradiol, these chemicals mimic the action of estrogen and may adversely affect normal reproductive processes for medaka fish and frog [2–4,7,12,21].

Bisphenol A (BPA), widely used as a monomer of polycarbonate plastics and a constituent of epoxy and polystyrene resins in industry and dentistry, is a non-steroid chemical and is also known to act as a xenoestrogen [2,12,13]. BPA has been detected in the liquid of canned vegetables and in the saliva of patients treated with dental sealants [1,20]. Therefore, humans as well as animals including wildlife are at risk of BPA exposure.

Various animal and cell culture studies have reported that BPA exhibits weak estrogenic activity. For instance, BPA can bind estrogen receptors, stimulate proliferation of the MCF-7 cell line, induce estrogen-responsive gene expression, increase the synthesis and release of prolactin, affect reproductive tissue morphogenesis, and alter behavior [6,9,13,14,22,23,25]. Recently, vitellogenin, synthesized as a consequence of estrogen-dependent gene expression, has been studied as a marker of

☆ *Abbreviations:* BPA, bisphenol A; E2, 17β-estradiol; ER, estrogen receptor; HE, hematoxylin and eosin; TUNEL, TdT-mediated dUTP-biotin nick end labeling.

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estrogenic activity. It was found that BPA-induced vitellogenin mRNA and protein expression in males [10,11]. However, these studies were mainly focused on whether BPA showed estrogenic activities, and few reports have examined whether BPA has an impact apart from estrogenic activity and what effect it has on non-reproductive tissues.

Embryogenesis is a critical stage in normal morphogenesis, with tissues such as the central nervous system and heart dynamically formed during a short period. Thus, the embryo is considered to be more fragile and sensitive to environmental factors compared to the fetus or adult [24]. However, the effects of xenoestrogens on early developmental stages remain unclear.

The aim of our study was to elucidate the effects of BPA on the early development of *Xenopus laevis* embryos. *X. laevis* is a suitable model to study the effects of endocrine disruptors, as eggs can be obtained throughout the year and developmental processes can be observed over a relatively short period of time [11,14,19].

Our results showed that BPA-exposed embryos exhibited marked malformations and specific apoptosis of central nervous system cells. Also, the effects of BPA appeared to be due to non-estrogenic activities.

Materials and methods

Chemicals. Bisphenol A (BPA) and 17β-estradiol (E2) were purchased from Kanto Chemical (Tokyo, Japan) and Wako Pure Chemical Industries (Osaka, Japan), respectively. L-Cysteine-HCl and Nonidet P-40 (NP-40) were purchased from Nacalai Tesque (Kyoto, Japan), and hematoxylin monohydrate and eosin G were purchased from MERK (Tokyo, Japan). Other chemicals were purchased mainly from Sigma (Tokyo, Japan) or Wako Pure Chemical Industries (Osaka, Japan).

Preparation of embryos. Unfertilized eggs were obtained from *X. laevis* females injected with 250 U gonatoropin (Teikoku Hormone MGF, Tokyo, Japan), a gonadotropic hormone. Eggs were fertilized, dejellied with a 2.5% solution of cysteine-HCl (pH 8.0), and developed in 10% Steinberg's solution (pH 7.4) at 21 °C until exposed to chemicals. *X. laevis* embryo development was staged according to Nieuwkoop and Faber [18].

Exposure of embryos to BPA and E2. Bisphenol A (BPA) and 17β-estradiol (E2) were dissolved in ethanol and diluted in 10% Steinberg's solution. The final concentration of ethanol in the solution was 0.2%. Embryos were exposed to BPA or E2 until the early tadpole stage when they had reached stage 6 or late 10. Control embryos were developed in solutions containing ethanol alone without BPA or E2.

Histological analysis. Control and exposed embryos were harvested at stages 19, 23, 33/34, and 40, fixed according to methods of Mizuhira and Hasegawa [16], and embedded using the JB4 kit (Poliscience, PA) (check spelling) according to manufacturer's instructions. Sagittal and cross sections were cut 10 or 4 μm thick, respectively, and stained with hematoxylin and eosin (HE) or TUNEL (TdT-mediated dUTP-biotin nick end labeling) [5]. TUNEL staining was carried out using an In Situ Cell Death Detection Kit, POD (Roche Diagnostics K.K., Tokyo, Japan) according to manufacturer's instructions. TUNEL-stained sections were counterstained with methyl green.

Isolation of genomic DNA. Ten embryos were harvested at the tail bud stage (stages 35/36, 37/38, and 40) and homogenized in lysis buffer (50 mM Tris–HCl, pH 7.5, 20 mM EDTA, and 1% NP-40). After centrifugation, the supernatants were collected and treated according to the apoptosis ladder detection kit (WAKO, Tokyo, Japan) method. Proteinase K (Takara, Tokyo, Japan) was added to a final concentration of 200 μg/ml and samples were incubated at 50 °C for 8–12 h. Samples were then treated with 100 μg/ml RNase A and extracted with phenol and then phenol:chloroform (1:1). DNA was precipitated with 3/5 volumes of 2-propanol in 2 M ammonium acetate and then redissolved in sterilized water. Total genomic DNA was stained with SYBR green I (Molecular Probes, OR) and visualized using a Lumi-Vision PRO (Aisin Seiki, Aichi, Japan).

Results

Effects of BPA on early developmental stages

To elucidate the effects of BPA on early development, *X. laevis* embryos were exposed for various periods to 10–100 μM BPA in 10% Steinberg's solution from developmental stage 6 to early tadpole stage. While normal control (non-exposed) embryos and embryos exposed to 10 μM BPA until the early tadpole stage showed normal development, embryos exposed to 40–100 μM BPA rapidly died during the gastrula stage (data not shown). The developmental abnormalities observed in early-stage embryos occurred when the embryos were exposed to as little as 20 μM BPA (Fig. 1). Although there was no difference between exposed and control embryos in terms of survival until stage 40 (data not shown), various abnormalities were observed in all exposed embryos, including: (i) neural tubes had not closed in any of the exposed embryos at stage 19 (Figs. 1A and E) but were completely closed in stage 19 control embryos; (ii) exposed embryos showed morphological defects at stages 23 (Figs. 1B and F) and 33/34 (Figs. 1C and G); and (iii) during the period corresponding to stage 40 in control embryos, embryos exposed to 20 μM

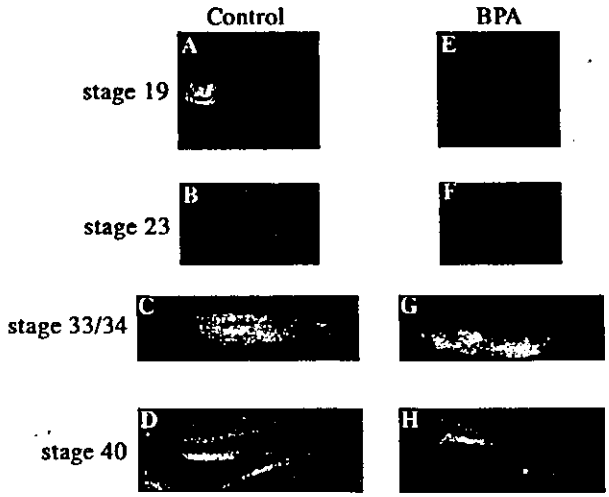

Fig. 1. Developmental abnormalities in early *X. laevis* embryos. Control and 20 μM BPA-exposed embryos are shown in (A–D) and (E–H), respectively. (A,E), stage 19; (B,F), stage 23; (C,G), stage 33/34; and (D,E), stage 40. The arrow indicates incomplete development of the neuronal tube.

BPA exhibited abnormalities such as crooked vertebrate, swollen abdomen, and malformation of the head (Figs. 1D and H). After stage 40, exposed embryos continued to develop with malformed heads and closed mouths, and died during the early tadpole stage.

Histological analysis of BPA-exposed embryos

Sections from control and BPA-exposed embryos were prepared for histological analysis and stained with hematoxylin and eosin (HE). In embryos exposed to 20 μM BPA from stage 6, cells with typical apoptotic features, in which the cell and its nucleus shrank and condensed, were identified at stages 33/34 and 40. Interestingly, these apoptotic cells were observed mainly in the prosencephalon, with some apoptotic cells having dropped into the brain ventricle spaces (Fig. 2). Similar observations were confirmed in the mesencephalon, rhombencephalon, and spinal cord, but not in other tissues (data not shown). Thus, apoptotic cells induced by 20 μM BPA were localized specifically to the central nervous system, whereas such apoptotic cells were not observed in control embryos (Fig. 2) or 10 μM BPA-exposed embryos (data not shown).

After finding that BPA affected embryo development, we then tested embryos at stages 10, 19 or 23. Stage 10 and higher embryos developed normally after BPA exposure, similar to controls. Histological analysis revealed that central nervous system cells also developed normally in these embryos (data not shown).

The detection of BPA-induced apoptosis

The presence of apoptosis was also assessed by TUNEL staining [5,26]. Sections were prepared from control and 20 μM BPA-exposed stage 40 embryos and stained using TUNEL. Exposure to 20 μM BPA strongly induced

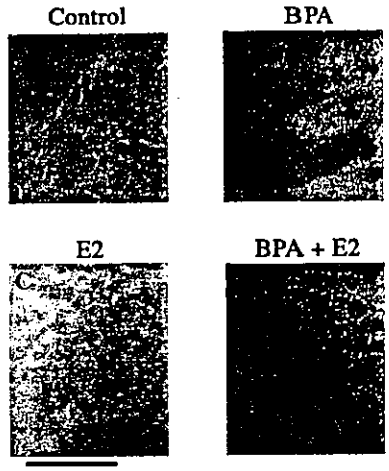

Fig. 3. Sagittal sections of the prosencephalon from stage 40 embryos stained with TUNEL. Control embryo (A) and embryos exposed to 20 μM BPA (B), 10 μM E2 (C), and both 20 μM BPA and 5 μM E2 (D). TUNEL-positive cells (apoptotic cells) are stained brown. The arrows indicate typical TUNEL-positive cells. Bar = 0.05 mm.

TUNEL-positive reactions in the prosencephalon (Fig. 3B) and brain ventricles (data not shown), similar to where the morphologically apoptotic cells were observed in HE-stained sections (Fig. 2). In contrast, no TUNEL positive cells were seen in control embryos. We also examined the appearance of 180-bp DNA ladders, a pattern indicative of apoptosis. Genomic DNA was extracted from 10 control and 20 μM BPA-exposed embryos. DNA ladders were visualized by polyacrylamide gel electrophoresis and SYBR green I staining. As shown in Fig. 4, DNA ladders were clearly detected only in embryos exposed to 20 μM BPA, especially in stage 40 embryos.

The effects of estrogenic activities on early development

To examine whether the effects caused by BPA were due to estrogenic action, stage 6 embryos were exposed

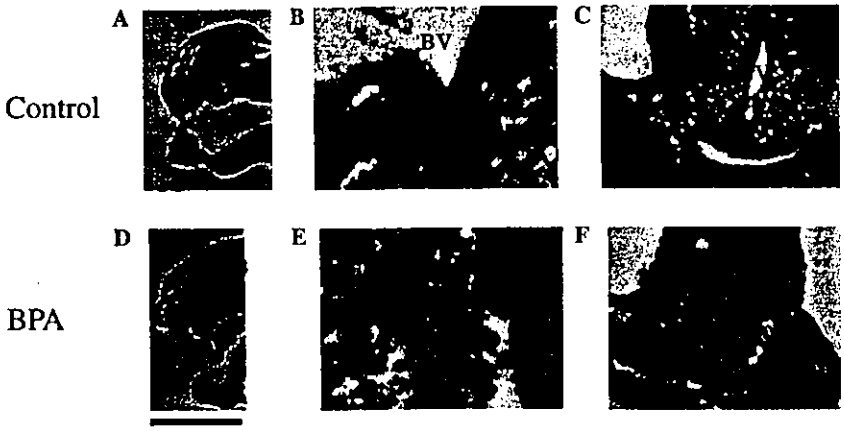

Fig. 2. Stage 40 sagittal and cross sections stained with HE. Sections from a control embryo and an embryo exposed to 20 μM BPA are shown in (A–C) and (D–F), respectively. (A,D) show a sagittal section of the head. Sagittal sections of the prosencephalon are shown in (B,E), and cross sections of the prosencephalon are shown in (C,F). FB, prosencephalon; MB, mesencephalon; BV, brain ventricle; OV, oral ventricle; and CG, cement gland. The arrow indicates cell morphology typical of apoptosis. Bar = 0.5 mm (A,D), 0.05 mm (B,E), and 0.1 mm (C,F).

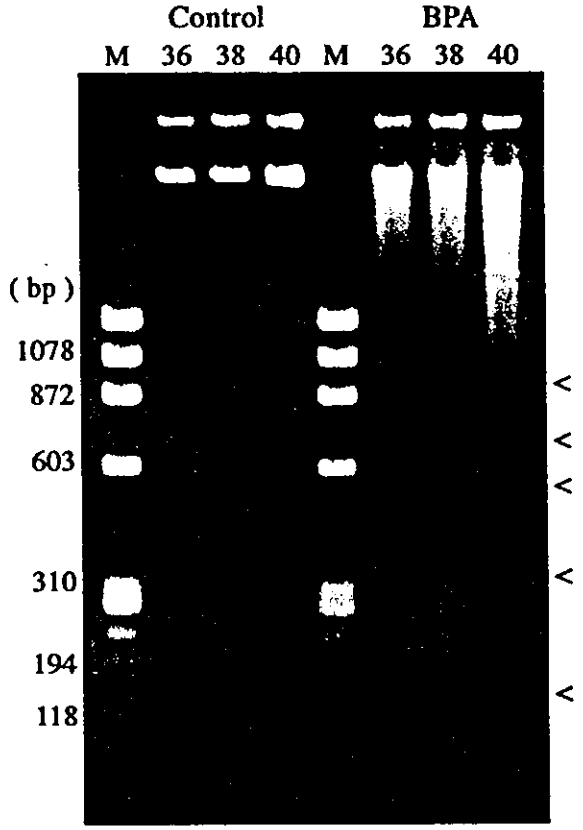

Fig. 4. DNA fragmentation analysis of BPA-exposed embryos. DNA was prepared from embryos at stages 36, 38, and 40 exposed by 20 μM BPA (BPA) or vehicle alone (control). Lane M, DNA size marker. The arrowheads indicate 180-bp DNA ladder bands.

to 1 or 10 μM E2. Similar to embryos exposed to 10 μM BPA, embryos exposed to 1 μM E2 appeared normal (data not shown). Likewise, embryos exposed to 10 μM E2 showed no abnormalities until stage 33/34. However, at stage 40, the malformations exhibited by these embryos differed partially from those embryos exposed to 20 μM BPA (Fig. 5). Marked malformations were observed in the head, abdomen, and axis of 10 μM E2-exposed embryos, as shown in Fig. 5. However, in contrast to BPA-exposed cells, histological sections prepared from stage 40 control or 10 μM E2-exposed embryos and stained with HE or TUNEL revealed no specific apoptosis of central nervous system cells (Figs. 3 and 6).

Simultaneous exposure to BPA and E2

To examine whether the BPA-induced malformations and specific apoptosis in central nervous tissue cells were inhibited by E2, embryos were simultaneously exposed to 1–10 μM E2 and 20 μM BPA. After exposure to 20 μM BPA and 10 μM E2 from stage 6, all embryos had died by stage 10. However, after exposure to 20 μM BPA and 1 or 5 μM E2, embryos showed developmental abnormalities typical of embryos exposed to BPA alone

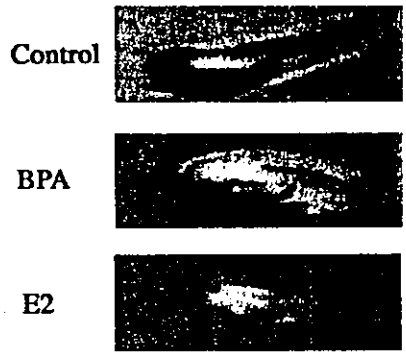

Fig. 5. Embryos exposed to 20 μM BPA or 10 μM E2. BPA- and E2-exposed embryos compared with control embryos at stage 40. (A) Control embryo; (B) 20 μM BPA-exposed embryo; and (C) 10 μM E2-exposed embryo.

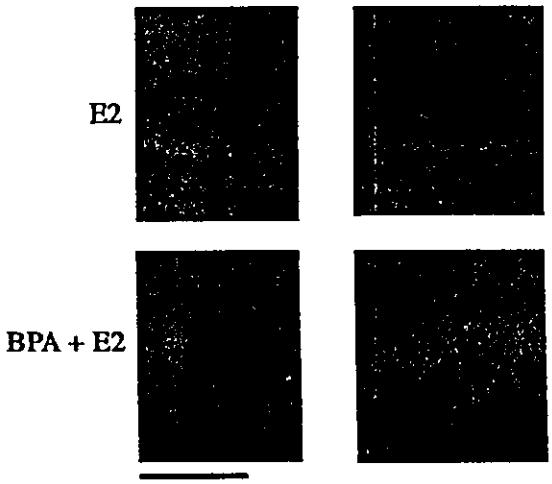

Fig. 6. Sagittal sections from stage 40 embryos stained with HE. Sections shown in (A,B) are from embryos exposed to 10 μM E2, sections shown in (C,D) are from embryos exposed to 20 μM BPA and 5 μM E2. (A,C) show the head sections, while (B,D) show prosencephalon sections. FB, prosencephalon; MB, mesencephalon; OV, oral ventricle; and CG, cement gland. The arrow indicates morphology typical of apoptotic cells. Bar = 0.5 mm (A,C) and 0.05 mm (B,D).

(data not shown). Sections from these embryos at stage 40 were prepared, and HE and TUNEL staining was performed to determine whether E2 inhibited the apoptosis of central nervous tissue cells. As shown in Figs. 2, 3, and 6, roughly the same numbers of apoptotic cells were induced in embryos exposed to BPA alone as were induced in embryos exposed to both BPA and E2. Thus, inhibition of apoptosis by the presence of E2 was not observed.

Discussion

Xenopus laevis has been used as an animal model to study the effects of environmental endocrine disrupters [11,14,19], including the effects of endocrine disruptors

on metamorphosis and sex differentiation in *X. laevis* larvae [8,11]. *X. laevis* embryos are also useful tools to observe the development of various tissues, including reproductive organs. Due to its crucial role in normal development it is thought that embryogenesis may be more fragile and sensitive to environmental compounds than development and growth of the fetus or adult [24]. However, the effects of xenoestrogens on early development remain unclear. In this study, we investigated the effects of BPA on early development using *X. laevis* embryos.

BPA-induced malformations and apoptosis of central nervous tissue cells during the early development of X. laevis embryos

Developmental abnormalities and specific apoptosis of central nervous tissue cells occurred when embryos were exposed to 20 μM BPA from stage 6. However, this phenomenon was not observed when the embryos greater than stage 6 were exposed to BPA, such that stage 10 embryos exposed to BPA exhibited normal morphogenesis and apoptosis. Thus, BPA appeared to adversely affect developmental processes between stage 6 and 10. BPA-induced apoptosis was localized specifically to central nervous tissue cells, especially in the brain, and was not detected in other tissues. This suggested that the central nervous system, including the brain, may be the target tissue for BPA during early embryo development, such that nerve cells appear to suffer adverse effects due to BPA during a period of cell differentiation and proliferation. In the present study, although apoptosis was not detected in control embryos, exposure to 20 μM BPA-induced apoptosis in the central nervous system (Figs. 2 and 3). Further studies are required to determine whether this abnormal apoptosis is due to the failure of normal differentiation.

The effects by BPA are caused by mechanisms other than through estrogenic activity

To examine whether the effects of BPA were mediated via estrogenic pathways, we exposed embryos to BPA and E2. As shown in Fig. 1, 20 μM BPA-exposed embryos showed developmental abnormalities at stage 19. However, embryos were not affected by exposure to 10 μM E2 until stage 33/34, with some abnormalities not appearing until stage 40. The occurrence of abnormal development of *X. laevis* embryos after exposure to 10 μM E2 has been previously reported, and the results of our present study are in general agreement with those obtained in previous studies (Fig. 5). E2-mediated abnormalities were not appeared until the tail bud stage (data not shown). These abnormal forms were partially similar to the morphological alterations observed in BPA-exposed embryos (Figs. 1 and 5). It has also been shown that the estrogenic activities of BPA generally are 1000–10,000 times less potent than those of E2 in terms of receptor binding assays using cultured human mammary cells (MCF-7), the estrogen-sensitive Fischer 344 rats or ovariectomized mice [13,15,17,20,22]. Similarly, the binding affinity of BPA to amphibian estrogen receptors has been reported to be approximately 700-times less potent than E2 by radio-receptor assays using liver cytosol fractions [14]. BPA-exposed embryos showed abnormal forms at concentrations of 20 μM, which was twice the concentration required for E2. Moreover, BPA induced specific apoptosis of central nervous tissue cells (Figs. 2 and 3), which was not observed in embryos exposed to E2 (Figs. 3 and 6). Some reports have found that E2 inhibited apoptotic cell death via estrogen receptor-mediated pathways in nerve cells [27]. In the present study, embryos exposed to 20 μM BPA exhibited nerve cell apoptosis that was not inhibited by E2 (Figs. 3 and 6). These results suggested that BPA mediated its adverse effects independently of estrogenic pathways.

We showed that the apoptosis occurred specifically in central nervous tissue cells by exposure of BPA but not by E2 at the equivalent dose during a limited period of embryogenesis of *X. laevis*. Although the mechanism is unclear, it was not E2 but BPA that induced apoptosis. In this paper, it became clear that E2 and BPA cause different effects at the concentration used, even if these chemicals have been classified in the group of the endocrine disrupting chemicals. The concentration of BPA used in this experiment is higher than that in general environment condition, however, it is conceivable that embryos might be exposed to the high concentration of chemical compound under some particular conditions. In such a case, our results obtained here are thought to provide useful information.

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Effects of chlorine on the decrease of estrogenic chemicals

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Abstract

The effects of chlorination on the elimination of three estrogenic chemicals such as 17β-estradiol, nonylphenol and bis-phenol A were investigated using yeast two-hybrid assay (YTA), estrogen receptor (ER) competition assay (ER-CA), and high-performance liquid chromatography/mass spectrometry (LC/MS). The results of YTA, ER-CA and the analysis of LC/MS indicated that the estrogenic activity of the above-mentioned three endocrine disruptors were significantly reduced as a result of chlorination. The decrease in estrogenic activity paralleled a decrease in estrogenic chemicals under the influence of free chlorine. One common characteristic of estrogenic chemicals is the presence of a phenolic ring. Considering that a phenolic ring is likely to undergo some sort of transformation in an aqueous chlorination solution, the above-mentioned results may be applied to the rest of the estrogenic chemicals in natural waters.

Keywords: Estrogenic chemicals; Yeast two-hybrid assay; Liquid chromatography/mass spectrometry; Estrogen receptor competition assay; Chlorination

1. Introduction

A number of chemicals exist in the environment that affect the hormonal system and produce an adverse effect on aquatic life, animals and probably on human as well [1–4]. Accordingly, there has been a heightened concern regarding the role of estrogenic chemicals, often referred to as environmental estrogens, in contributing to the development of hormone-dependent cancers, disorders of the reproductive tract, and other effects [1–4]. Estrogenic chemicals are a diverse group of endocrine disruptors that do not always share any chemical structural resemblance to the prototypical estrogen, 17β-estradiol (E2), but evoke agonist or antagonist responses possibly through a comparable mechanism of action [5]. As a result, estrogenic chemicals can be identified by their ability to bind to the estrogen receptor (ER) and to induce an estrogenic response. Although phthalate esters, hydroxylated polychlorinated biphenyls (PCBs), pesticides, and a number of complex mixtures have been shown to contain estrogenic activities [6,7], preliminary studies conducted so far indicated that three major estrogenic chemicals identified in the effluents from sewage treatment plants and river waters were E2, nonylphenol (NP) and bis-phenol A (BPA) [8,9]. Some of these river waters have been used as source waters for water supply. Although disinfection with chlorine is still one of the major processes of the rapid sand filtration system, there has been a concern that disinfection with chlorine, while providing protection against microbial risks, could also pose chemically induced cancer risks due to the formation of the trihalomethanes (THMs) and other carcinogenic disinfection by-products (DBPs) such as dichloroacetic acid. However, little is known regarding

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the estrogenic effects of disinfection with chlorine on the estrogenic potency. Thus, this research was conducted by use of yeast two-hybrid assay (YTA) (with rat ER and the co-activator, TIF2) [10] to identify whether or not disinfection with chlorine eliminates or more strongly induces the estrogenic potency of E2, NP and BPA. To assess whether the estrogenic compounds identified by YTA are likely to show a similar receptivity to other receptors such as human ER, estrogen receptor-competition assay ER-CA was also conducted. Because one common characteristic of estrogenic compounds is the presence of a phenolic ring, and considering that a phenolic ring is likely to undergo some sort of transformation in aqueous chlorine solution, chlorination results of BPA, NP and E2 may be applied to estimate the effect of disinfection with chlorine on the estrogenic potency of other environmental waters.

2. Methods

2.1. Chemicals tested

As mentioned above three estrogenic chemicals, BPA (Kanto Chemical Co., Inc., Tokyo, Japan), NP (Kanto Chemical Co., Inc., Tokyo, Japan), and E2 (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) were selected for this investigation. Test chemicals were made up in methanol to 10^{-1} M (BPA), 10^{-2} M (NP), 10^{-3} M (E2) stock solutions and stored at 4°C. Stock solutions were diluted with purified water just before chlorination. This gave a final concentration of 10^{-5} M for BPA, 10^{-6} M for NP, and 10^{-7} M for E2.

2.2. Chlorination

Sodium hypochlorite (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) was used as the chlorinating agent. Reaction with chlorine proceeded under the specified conditions in stoppered dark bottles without headspace. The reaction conditions such as chlorine dose are given in the corresponding figures. At the end of the desired reaction time the samples to be analyzed for YTA [10], and ECA [11] were quenched with ascorbic acid in excess of the stoichiometric amount. The chlorine concentration was measured by DPD colorimetric method [12].

2.3. Extraction of reaction products

Sep Pak C18 cartridge (Waters, USA) adsorption coupled with methanol elution was used to prepare extracts from the sample waters before and after chlorination. The recovered products were resolved with methanol before YTA, ER-CA, and liquid chromatography/mass spectrometry (LC/MS) analysis.

2.4. YTA [10]

The estrogenic activities of test chemicals were examined with YTA. This assay is based on the ligand-dependent interaction of two proteins, a hormone receptor (rat ER-α) and a co-activator (TIF2), and the hormonal activity is measured by the level of β-galactosidase activity. In this study, modified YTA was used because the original method could not detect the estrogenic activity in natural waters due to the hindrance of co-existing compounds in natural water [5]. The cells were pre-incubated overnight at 30°C in an SD medium. The culture in micro test tube was then mixed with a methanol solution of test samples and incubated for 4 h at 30°C. After washing by centrifugation, the cells were digested enzymatically by incubation with 1 mg/mL Zymolyase 20 T at 37°C for 15 min. The lysate was mixed with 4 mg/mL *o*-nitrophenyl-β-*D*-galactoside (ONPG) and reacted at 30°C for 30 min before the reaction was stopped by the addition of 1 M Na_2CO_3. An aliquot was taken into each of the 96 wells of micro plate. Absorbance at 420 nm for color and 570 nm for turbidity were read on a micro plate reader (BIO RAD, Model 550, USA) to estimate estrogenic activity. The results were evaluated as the relative estrogenic activity (estrogenic activity of the test sample to that of E2 (10^{-4} M) used as the positive control). The Proliferation rate of yeast was calculated with the following equation:

$$\text{Proliferation rate of yeast}(\%) = 100 \times \frac{(\text{absorbance at 595 nm after incubation for 4 h})}{(\text{absorbance at 595 nm before incubation})}. \quad (1)$$

2.5. ER-CA [11]

ER-α, β competitor assay kit (PanVera Inc., Madison, WI, USA) was used to observe competition for binding between fluorescence-labeled estrogen and compounds which possess estrogen potential such as NP, BPA and E2. This kit contains the reagents necessary to perform a competition assay to assess the affinity of test compounds for the human estrogen receptor-α, β. ER is added to a fluorescent estrogen (Fleuron ES2) ligand to form an ES2/ER complex with high fluorescence polarization. The complex is then added to estrogen competitor test compounds in micro titer wells. As shown below, the shift in polarization in the presence of test compounds is used to determine the relative affinity of test compounds for ER. Accordingly, the shift

in polarization for lower values indicates the presence of estrogenic compounds:

Test compounds + ES2/ER – α or β[high polarization]
→ Test compounds/ER – α or β
+ ES2[low polarization]. (2)

2.6. LC/MS analysis

LC/MS (HP1100MSD, USA) equipped with a diode array detector and mass spectrometer (Electrospray ionization mode) was used to identify and quantify the reaction product of E2, NP and BPA by chlorination. Reverse-phase LC/MS was performed with 2.1 mm × 150 mm columns packed with 5-μm ZORBAX Eclipse XDB-C18 (HP, USA). Gradient elution was started mainly at 50% (volume percent) 0.1 mM sodium formate solution and programmed to 100% methanol in a linear manner program at a flow rate of 0.2 mL/min over a period of mainly 30 min. LC/MS analysis was performed by electrospray ionization mode. A fraction collector (Advantec type SF-3120, Advantec Toyo, Tokyo, Japan) was used to collect the characteristic reaction products formed as a result of chlorination.

3. Results and discussion

3.1. Effects of chlorination time on the estrogenic activity and toxicity

The effects of chlorination time on the elimination of estrogenic activity of BPA, NP, and E2 are presented in Figs. 1–3. Elimination of estrogenic potency is highly reaction time dependent. No significant decrease in estrogenic activity of NP and E2 was obtained in 10 min due to the relatively low dose of free chlorine (1.5 mg/L). In contrast, in the case of BPA, chlorination for 10 min was sufficient enough to reduce estrogenic potency due

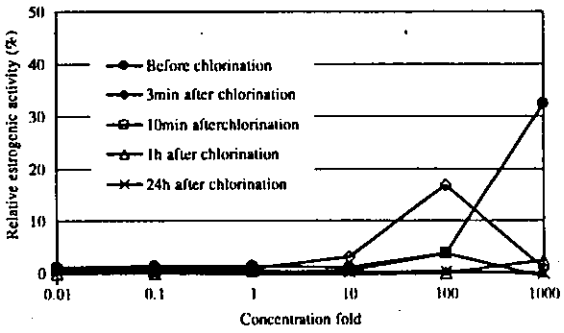

Fig. 1. Effect of chlorination time on the elimination of estrogenic activity induced by BPA (10^{-5} M) (chlorine dose: 7.5 mg/L, pH: 7.0, temperature: 20°C).

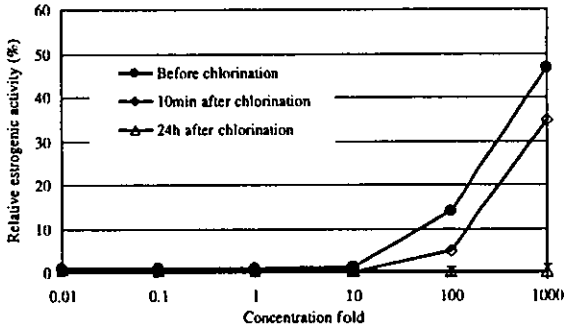

Fig. 2. Effect of chlorination time on the elimination of estrogenic potential induced by NP (10^{-6} M) (chlorine dose: 1.5 mg/L, pH: 7.0, temperature: 20°C).

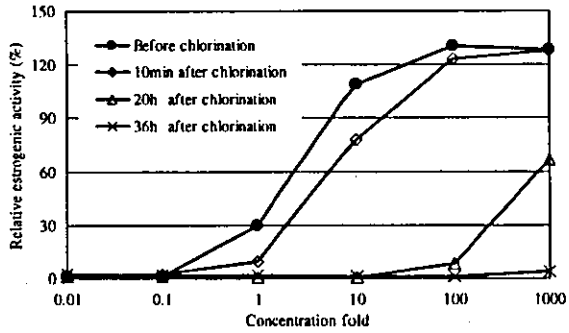

Fig. 3. Effect of chlorination time on the elimination of estrogenic potential induced by E2 (10^{-7} M) (chlorine dose: 1.5 mg/L, pH: 7.0, temperature: 20°C).

to the relatively high dose of free chlorine (7.5 mg/L) as shown in Fig. 1. An apparent decrease in estrogenic potency after a 3 min reaction time at concentration fold (volume of raw water/volume after adsorption and desorption with solvent) of 1000 is due to the inhibition of yeast proliferation by the toxic effect of the reaction by-products (Fig. 4). As shown in Fig. 5, it is evident that no impurities brought about by the addition of chlorine was responsible for the estrogenic activity or the inhibition of yeast proliferation. Chlorination of BPA for 3 min results in the formation of estrogenic chemicals with a little bit greater estrogenic potency than the initial BPA. The estrogenic potency which emerged after 3 min was not stable in the presence of free chlorine and dissipated with the extended chlorination. These results indicate that these toxic products were formed as a result of insufficient chlorination (insufficient chlorine dosage with insufficient reaction time). Complete elimination of estrogenic activity of E2 with initial chlorine level of 1.5 mg/L required longer than 36 h at any level of concentration fold. Critical $C \times T$ (concentration of free chlorine multiplied by reaction time) was not found at

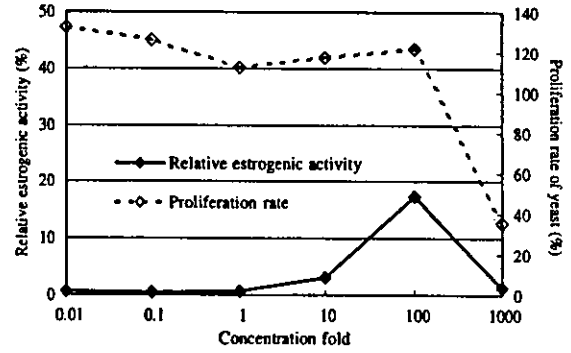

Fig. 4. Relative estrogenic activity and yeast proliferation after 3 min chlorination of BPA (10^{-5} M) (chlorine dose: 7.5 mg/L, pH: 7.0, temperature: 20°C).

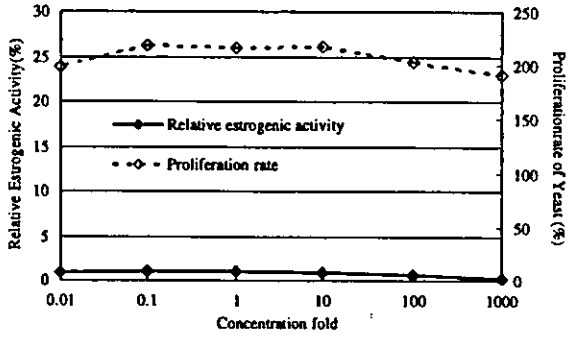

Fig. 5. Relative estrogenic activity and yeast proliferation after 3 min chlorination (7.5 mg as Cl/L, for 3 min) of purified water used for spiking BPA (10^{-5} M).

this moment; however, it is evident that the decrease of both estrogenic activity and toxicity is proportional to $C \times T$.

Both oxidation of organics and substitution of chlorine on organic carbon to form chlorinated organic compounds are known to occur with free chlorine. At this moment it is impossible to specify which one between oxidation reaction and substitution reaction has the dominant effect on reducing the estrogenic activity induced by E2, BPA, or NP. However, a previous study [9] regarding the effect of ozonation on the elimination of the estrogenic activity has suggested that the reduction in the estrogenic activity induced by E2, BPA, or NP may be mainly attributed to the oxidation of E2, BPA, or NP.

Considering that most estrogenic chemicals contain a phenolic ring in their chemical structure and the phenolic ring is likely to undergo some sort of oxidation in aqueous chlorination solution, almost all the estrogenic chemicals found in natural waters may be decomposed as a result of oxidation by the addition of free chlorine (around 1–7 mg/L) used in the water treatment plant.

3.2. ER-CA

To confirm the decrease in estrogenic activity as a result of chlorination, ER-CA human ER-α, or -β with competition assays were conducted after chlorination and compared with those of BPA, NP, and E2 before chlorination. Because ER-CA with rat ER-α was not available during the course of this research, ER-CA with human ER-α was used to evaluate the affinity of BPA, NP, and E2 to ER (human ER-α). Further research is necessary to reveal the difference in affinity for estrogen chemicals between human ER-α and rat ER-α. Preliminary tests indicated that endpoints obtained by the use of human ER-β were almost similar to those obtained by the use of human ER-α. Accordingly, only the results obtained by the use of ER-β are presented in this report (Figs. 6–8). As shown in Figs. 6–8, the affinity of BPA, NP, and E2 to ER also decreased as a result of chlorination, indicating the effectiveness of 24 h chlorination in reducing the estrogenic activity of BPA,

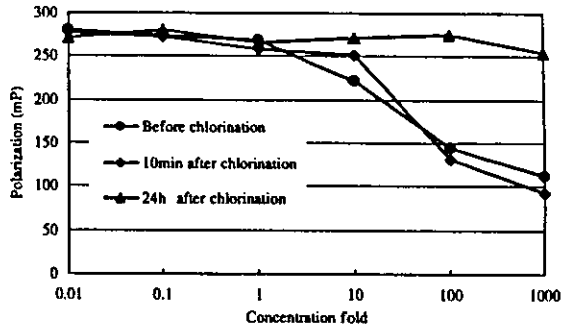

Fig. 6. Effect of chlorination time on the affinity of BPA to ER-β (chlorine dose: 7.5 mg/L, pH: 7.0, temperature: 20°C).

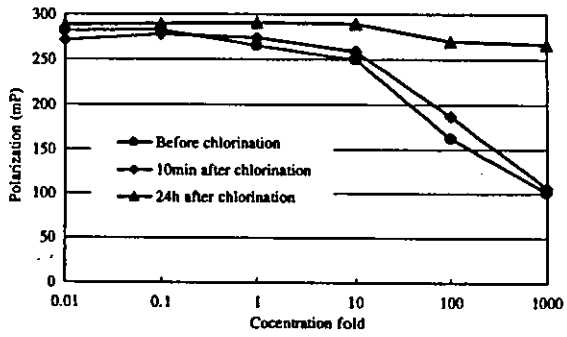

Fig. 7. Effect of chlorination time on the affinity of NP to ER-β (chlorine dose: 1.5 mg/L, pH: 7.0, temperature: 20°C).

NP, and E2. A slight decrease in polarization at a concentration fold of 100 and 1000 after 10 min chlorination of BPA (Fig. 6) indicates the slight increase in binding affinity between the ER and chlorination by-products. However, as presented in Fig. 1, almost no significant estrogenic activity was observed after 10 min chlorination. It appears that no significant increase in estrogenic activity is likely due to the toxic effects of chlorination by-products formed after 10 min chlorination of BPA as also similarly indicated by the poor yeast proliferation at a concentration fold of 1000 after 3 min chlorination of BPA (Fig. 4). However, Cl_1–Cl_3 compounds (which may be responsible for the toxic effect on yeast cells) formed after 3 min chlorination were destroyed after 10 min chlorination (Table 1). Consequently, the most plausible explanation at this stage for the low estrogenic activity at a concentration fold of 1000 after 10 min chlorination of BPA may be due to the formation of chlorination by-products which can bind to the yeast cell's ER, but cannot induce transcriptional activation.

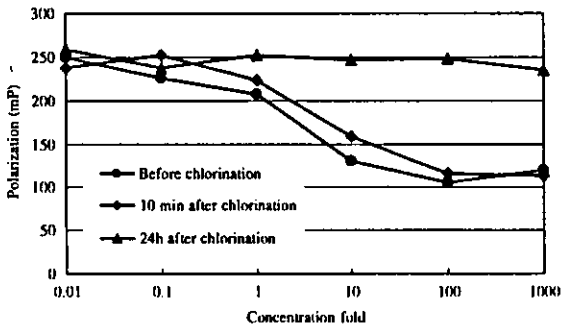

Fig. 8. Effect of chlorination time on the affinity of E2 to ER-β (chlorine dose: 1.5 mg/L, pH: 7.0, temperature: 20°C).

3.3. Chlorination products

Typical LC/MS chromatograms before and after chlorination for BPA are revealed in Figs. 9 and 10, respectively. The reaction products formed as the results of chlorination of BPA, NP, and E2 and separated by LC/MS are illustrated in Tables 1–3. These tables list the retention times of the compounds for each major peak together with quasi-molecular ions $(M\text{-}H)^-$ (*m/z*) of the compounds.

These data also support the decrease in estrogenic potency and parallel a decrease in estrogenic chemicals under the influence of free chlorine. In the case of NP and BPA, some of the reaction products such as trichloro-phenol (*m/z* 197) persisted even after 24 h. However, no estrogenic response was seen in these chlorination products of BPA, NP, and E2 after 24 h.

As shown in Table 3 the reaction time of 10 min was not sufficient enough to decompose E2. The *m/z* 271 quasi-molecular ion $(M\text{-}H)^-$ observed in the electrospray ionization spectra of E2 was also observed after 10 min, indicating that E2 was not completely decomposed in 10 min. Accordingly, it follows that the

Table 1
Retention time and quasi-molecular ion or fragment ions of reaction products formed as a result of chlorination of BPA (10^{-5} M) (chlorine dose: 7.5 mg/L, pH: 7.0, temperature: 20°C)

Sample	Concentration fold[a]	Retention time (min)	*m/z*	Abundance	Assignment
Before chlorination	1	8.87	227	92,000	BPA
3 min after chlorination	1	10.33	261	65,000	Cl_1[b]
		11.29	295	380,000	Cl_2[b]
		12.06	329	290,000	Cl_3[b]
		12.74	365	280,000	Cl_4[b]
10 min after chlorination	1	12.86	331	21,000	
		13.71	365	400,000	Cl_4[b]
		16.12	525	58,000	
		16.12	253	60,000	
		16.12	262	22,000	
1 h after chlorination	1	12.73	365	265,000	Cl_4[b]
		14.66	525	135,000	
24 h after chlorination	10	11.25	195	75,000	
			197	66,000	2,4,6-TCP
		17.72	336	68,000	

[a] Volume of raw water/volume after adsorption and desorption with solvent.
[b] The numerical suffix in Cl_1–Cl_4 indicates the number of chlorine atoms present in chlorinated BPA.

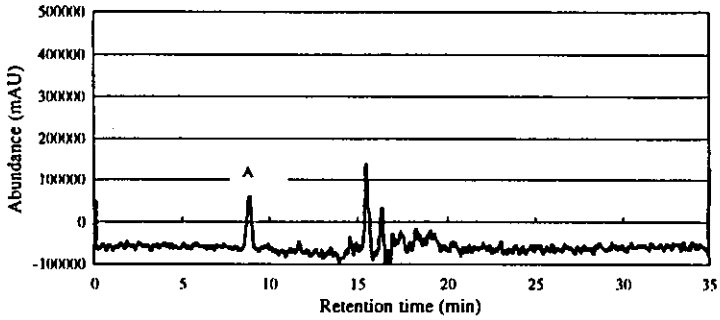

Fig. 9. Total ion (TI) chromatogram for BPA (10^{-5} M) before chlorination (Peak A indicates the presence of BPA).

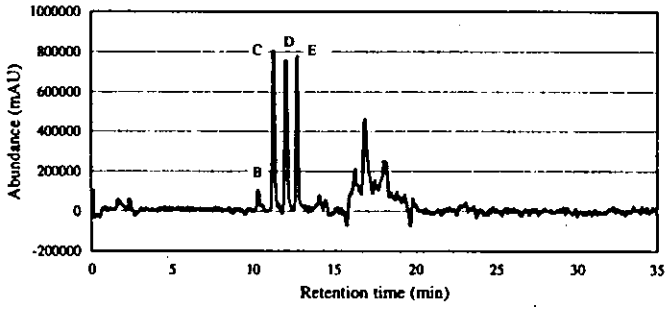

Fig. 10. TI chromatogram for BPA (10^{-5} M) after chlorination (Peaks B, C, D, E indicate the formation of chlorination by-products from BPA) (chlorine dose: 7.5 mg/L, pH: 7.0, temperature : 20°C).

Table 2
Retention time and quasi-molecular ion or fragment ions of reaction products formed as a result of chlorination of NP (10^{-6} M) (chlorine dose: 1.5 mg/L, pH: 7.0, temperature: 20°C)

Sample	Concentration fold[a]	Retention time (min)	*m/z*	Abundance	Assignment
Before chlorination	1	20.86	219	220,000	NP
10 min after chlorination	10	20.86	219	135,000	NP
		21.72	253	86,000	Cl_1[b]
		22.62	287	120,000	Cl_2[b]
24 h after chlorination	10	7.38	237	24,000	
		9.09	197	45,000	2,4,6-TCP
			195	20,000	
		9.36	225	29,000	

[a] Volume of raw water/volume after adsorption and desorption with solvent.
[b] The numerical suffix in Cl_1–Cl_4 indicates the number of chlorine atoms present in chlorinated NP.

estrogenic activity induced after 10 min is due to the unreacted E2 with chlorine. As indicated in Table 3, the *m/z* 355 and 401 ions found after 20 h and 36 h are caused by the chlorination product of E2.

4. Conclusions

- In a study of the reaction of chlorine with E2, NP, and BPA, it was concluded that chlorine is an active agent for eliminating the estrogenic activity.
- The decrease in estrogenic potency parallels a decrease in estrogenic chemicals under the influence of free chlorine probably due to the oxidation effect of the chlorination.
- The result of this research may be applied to the rest of the estrogenic chemicals because the rest of the estrogenic compounds also contain a phenolic ring that is likely to be oxidized by chlorine.
- These findings present another problem as being a trade-off between the beneficial side of chlorination, which is a reduction in estrogenic activity or

Table 3
Retention time and quasi-molecular ion or fragment ions of reaction products formed as a result of chlorination of E2 (10^{-7} M) (chlorine dose: 1.5 mg/L, pH: 7.0, temperature: 20°C)

Sample	Concentration fold[a]	Retention time (min)	*m/z*	Abundance	Assignment
Before chlorination	1	10.06	271	82,000	E2
10 min after chlorination	100	10.10	271	18,000	E2
		10.58	355	65,000	
		12.71	305	85,000	
		13.10	369	60,000	
20 h after chlorination	100	9.93	355	200,000	
			401	100,000	
		11.60	389	28,000	
36 h after chlorination	100	10.44	355	225,000	
			401	105,000	

[a] Volume of raw water/volume after adsorption and desorption with solvent.

germicidal effect, and the disadvantageous side of chlorination, which is an increasing formation of chlorination by-products such as chloro-phenol. Further study is necessary to identify the critical level of residual chlorine together with the minimum required reaction time for the elimination of the estrogenic activity of source water.

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Products of Aqueous Chlorination of 17β-Estradiol and Their Estrogenic Activities

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To assess the estrogenic activity potentially stemming from 17β-estradiol (E2) in drinking water, ESI-LC-MS was used to identify the products of its aqueous chlorination under the following conditions: 50 μg/L E2, 1.46 mg/L sodium hypochlorite, pH 7.5, 25 °C. Seven products, including 2,4-dichloro-17β-estradiol, monochloroestrone, 2,4-dichloroestrone, and the four byproducts such as 4-[2-(2,6-dichloro-3-hydroxyphenyl)ethyl]-7α-methyloctahydroinden-5-one (product C in the text) were identified in chlorinated E2 solution. The estrogenic activities of the aqueous chlorinated E2 solution at 10, 30, 60, 120, and 180 min contact time were assessed by a yeast two-hybrid system based on the ligand-dependent interaction of two proteins, a human estrogen receptor (ER) and a coactivator. All five solutions elicited transcriptional activation induction. The maximal β-galactosidase activities induced by the chlorinated solution at 10, 30, and 60 min were similar and slightly lower than those before chlorination, while the activities of the chlorinated solution at 120 and 180 min were about 40% of those before chlorination. Finally, 4-chloro-17β-estradiol (4-chloro-E2) (we failed to synthesize the 2-chloroestrone (2-chloro-E1)), 2,4-dichloro-17β-estradiol (2,4-dichloro-E2), and 2,4-dichloroestrone (2,4-dichloro-E1) were synthesized, and product C was fractionated by HPLC. It was found that 4-chloro-E2 elicited strong estrogenic activity, at almost the same level as that of estrone (EC50 = 10^2 nM), while 2,4-dichloro-E2 elicited weaker β-galactosidase activity compared with that of 4-chloro-E2. The EC50 was ca. 10^3 nM. The maximal β-galactosidase activity for 2,4-dichloro-E1 was lower than that of 2,4-dichloro-E2, while its EC50 was similar to that of 2,4-dichloro-E2. In addition, product C, 4-[2-(2,6-dichloro-3-hydroxyphenyl)ethyl]-7α-methyl-octahydroinden-5-one, induced high β-galactosidase activity at the relatively higher concentration of 3.5×10^5 nM. On the basis of the dose−response curve of a single byproduct of chlorinated E2, the estrogenic activity at 120 and 180 min appears to be induced mainly by 2,4-dichloro-E2 and 2,4-dichloro-E1.

Introduction

A number of papers have highlighted the potentially detrimental effects of some anthropogenic compounds on reproductive processes in wildlife and humans. There is increasing evidence that these compounds can alter endocrine function and may disrupt growth, development, and reproduction by interfering with the production, release, transport, metabolism, binding, action for elimination of endogehomeostasis, and regulation of developmental processes (*1*, *2*). Estrogenic effects on aquatic wildlife have been linked to some particular compounds, both man-made, such as nonylphenol and bisphenol A, and those occurring naturally (*3*).

Of concern is the endogenous hormone 17β-estradiol (E2). This compound is an extremely potent estrogen receptor modulator, and it has been reported that fish exposed to E2 exhibit changes in biomarkers for estrogenicity at concentrations as low as 0.5 ng/L (*4*−*7*). A laboratory experiment also supports a direct relationship between E2 exposure and inhibition of testicular growth in fathead minnows (*8*). A recent study has identified that E2 is one of the compounds responsible for the estrogenicity of effluents from seven sewage treatment plants in the U.K. (*9*). In addition, E2 together with other steroidal estrogens has been listed in the Tenth Report on Carcinogens (*10*).

The occurrence of E2 in the aquatic environment is due to the discharge of municipal wastewater (*11*−*14*). E2 levels between 2.5 and 3.5 μg are daily excreted by menstruating and menopausal women, respectively, while pregnant women may secrete up to 259 μg a day. For men, an E2 level of 1.6 μg/day is excreted in the urine (*15*). E2 is excreted mainly as conjugates of sulfuric and glucuronic acids. Although the E2 conjugates do not possess a direct biological activity (*16*, *17*), they can act as precursors of E2 reservoirs capable of being reconverted to free E2 by bacteria in the environment or sewage treatment works (*9*). Compound E2 is prevalent in the aquatic environment, adding to concerns about this chemical. Residues of E2 have been reported in river and tap water (*18*−*21*). While many papers highlight the occurrence of E2 in the aqueous environment, little attention has been paid to its behavior in water supply systems. It is well-known that disinfection by chlorination is an essential step in the treatment of water supply systems and that phenolic compounds are reactive with hypochlorite (*22*). In our previous paper, it was shown that the product of aqueous chlorination of bisphenol A exhibited greater estrogenic activity than the parent bisphenol A (*23*) and that some products in the chlorinated 4-NP solution elicited anti-estrogenic activities (*24*). Knowledge of the fate of E2 after chlorination is needed in order to allow effective assessment of the endocrine disruption potential of residual E2 in drinking water.

In this study, the aqueous chlorinated products of E2 were identified by an ESI-LC-MS method, and a pathway is proposed. The estrogenic activities of aqueous chlorinated E2 solutions were investigated using a yeast two-hybrid assay. Finally, some byproducts were synthesized and their estrogenic activities were also determined in order to examine which byproducts contribute to the estrogenic activities in aqueous chlorinated E2 solution.

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Material and Methods

Computational Chemistry. MOPAC (version 6) was used as adapted by CAChe Scientific Inc. (Oxford, U.K.) and carried out on an IBM 600E computer. The PM3 parameter (*14*) served to optimize stable structures. The program was used to obtain optimum geometries and atom partial charges.

Chlorination Procedures. The experiments were carried out in a glass reactor which was placed in a water bath to maintain the reaction temperature at 25 °C. The reactor was stirred during the contact period at 100 rpm. Synthetic raw water was prepared by dissolving 0.2 mg of a standard E2 or E1 into 4 L of Milli-Q pure water (50 μg/L), adjusted to pH 7.5 by phosphoric acid. The sample of 500 mL was removed for determination of the estrogenic activity before sodium hypochlorite (1.46 mg/L) was added to the remaining solution. Samples (500 mL) were taken out at 10, 30, 60, 120, and 180 min. After decomposition of the residual sodium hypochlorite by the addition of $Na_2S_2O_3$ and adjusting the pH to 2–3, the samples were concentrated by solid-phase extraction (Waters Sep-Pak C18, Waters,). The cartridge was conditioned with 5 mL of acetonitorile, 5 mL of methanol and 5 mL of water. Samples were passed through the cartridge at a flow rate of 10–15 mL/min. Additional water (10 mL) was applied to wash the wall of the cartridge. The residual water was removed by passing a gentle nitrogen stream through the cartridge for about 30 min. Dichloromethane (5 mL) followed by 5 mL of CH_3CN was percolated at 4–5 mL/min through the sorbent bed to mobilize E2 and its chlorinated products. Of the 10 mL of eluant, 8 mL was dried under a gentle nitrogen stream and redissolved in 0.2 mL of CH_3CN for ESI-LC/MS analysis. The rest of the eluant (2 mL) was redissolved in 0.02 mL of dimethyl sulfoxide (DMSO) to assess the estrogenic activity using the yeast two-hybrid assay.

E2 and E1 were purchased from Kanto Chemical Co. (Tokyo, Japan).

Characterization of Products. The products in an aqueous chlorinated solution of E2 and E1 were characterized by ESI-LC-MS. Liquid chromatography was performed on an Alliance 2490 HPLC (Waters) equipped with a quaternary gradient pump, an autosampler with a 100 μL injection loop at a flow rate of 0.2 mL/min. The analytes were chromatographed on a Diamonsil C_{18} (Dima) silica packed LC column (2.1 mm i.d., 15 cm in length, 5 μm in particle diameter) at 40 °C. The initial mobile-phase composition was acetonitorile/water (10/90 (v/v)), which was kept for 1 min, increased linearly to 50/50 in 15 min, kept for 10 min, finally, increased linearly to 100% in 5min, and kept for 5 min.

A platform ZMD single quadrupole mass spectrometer (Micromass, Manchester, U.K.) was used with a Z-Spray ion source fitted with a pneumatically assisted electrospray probe. E2, E1, and their products were detected in the negative mode; typical ion source parameters used were as follows: ESI capillary voltage at 2.5 kV; extractor voltage at 5 V; source block temperature at 130 °C; desolvation temperature at 400 °C; ion energy at 0.8 V; multiplier voltage at 650 V. Nitrogen was used as the desolvation gas with a flow of 500 L/h and the cone gas at a flow of 100 L/h; the cone voltage at 60 V with the full scan mass ranged from 200 to 800 with a scan time of 1.2 s. Masslynx 3.4 workstation software was used for data processing. The injection volume of the sample was 20 μL.

Estrogenicity of Products. The yeast two-hybrid assay was applied to evaluate the estrogenicity of aqueous chlorinated E2 and E1 solutions. The yeast two-hybrid assay system with the estrogen receptor, ERα, and the coactivator, TIF2, was used to investigate the transcriptional activation induced by the products of E2 and E1 formed during aqueous chlorination. This method has been described in a previous paper (*25*).

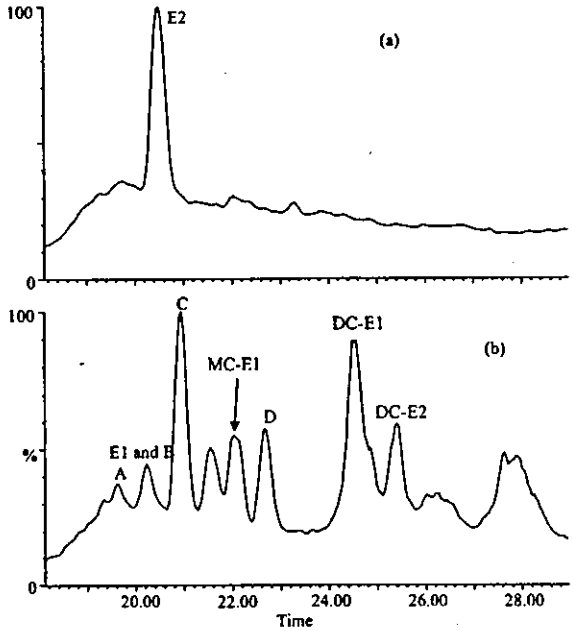

FIGURE 1. LC-MS chromatogram of chlorinated E2 solution: (a) before chlorination; (b) with chlorination (time, 10 min). E1, estrone; MC-E1, 2-chloroestrone; and 4-chloroestrone; DC-E1, 2,4-dichloroestrone; DC-E2, 2,4-dichloro-estradiol. A–D: unknown.

Synthesis of 4-Chloro-E2, 2,4-Dichlro-E2, and 2,4-Dichloro-E1. An aqueous solution of sodium hypochlorite (0.5%, 4 mL) was added to a stirred solution of E2 (100 mg) in tetrahydrofuran (10 mL). After stirring for 1 h, an aqueous solution of sodium sulfate was added. The solution was acidified with 10% hydrochloric acid, concentrated to half-volume under reduced pressure, saturated with sodium chloride, and then extracted with ether. The ethereal solution was washed with brine and dried over magnesium sulfate. After removal of the solvent, the residue was subjected to preparative HPLC (ODS-A, 50 mm × 250 mm, YMC Co. Ltd., Kyoto, Japan; 70% methanol) to give 2,4-dichlro-E2 (16 mg) and 2,4-dichloro-E1 (12 mg), and further purification of the other fractions by HPLC (ODS-A, 20 mm × 250 mm, YMC; 60% acetonitrile) gave 4-chloro-E2 (10 mg).

4-Chloro-E2. FAB-MS *m/z*: 306 (M^+, ^{35}Cl), 308 (M^+, ^{37}Cl). ^1H NMR ($CDCl_3$): δ 6.85 (1H, d, J = 8.7 Hz, 1-H), 7.17 (1H, d, J = 8.7 Hz, 2-H).

2,4-Dichloro-E2. FAB-MS *m/z*: 340 (M^+, $^{35}Cl + ^{35}Cl$), 342 (M^+, $^{35}Cl + ^{37}Cl$), 344 (M^+, $^{37}Cl + ^{37}Cl$). ^1H NMR ($CDCl_3$): δ 7.21 (1H, s, 1-H).

2,4-Dichloro-E1. FAB-MS *m/z*: 338 (M^+, $^{35}Cl + ^{35}Cl$), 340 (M^+, $^{35}Cl + ^{37}Cl$), 342 (M^+, $^{37}Cl + ^{37}Cl$).^1H NMR ($CDCl_3$): δ 7.21 (1H, s, 1-H).

Results and Discussion

Characterization of Products. Parts a and b of Figure 1 show the LC/MS chromatograms of chlorinated E2 before chlorination and after 10 min chlorination, respectively. E2 rapidly reacted with HOCl, and several chlorinated products were formed while no E2 was detected. The molecular ion typically appears in the LC/MS mass spectrum as described in our previous paper (*26*). The molecular ions at *m/z* 303 (one chlorine atom), 337 (two chlorine atoms), and 339 (two chlorine atoms) were observed in the mass spectra of three peaks at 22.14, 24.52, and 25.39 min, respectively (Figure 2). Although no fragment ions were found, the products corresponding to these peaks were postulated to be monochloro-E1, dichloro-E1, and dichloro-E2, based on the molecular

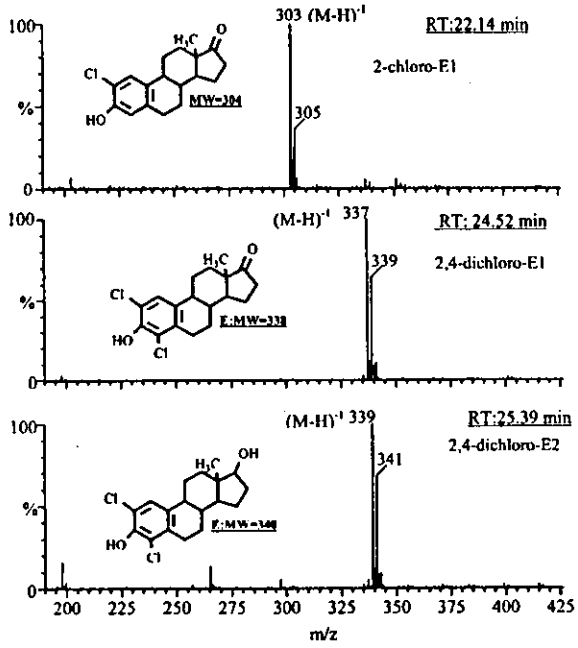

FIGURE 2. LC-MS mass spectra of 2-chloro-E1, 2,4-dichloro-E1, and 2,4-dichloro-E2.

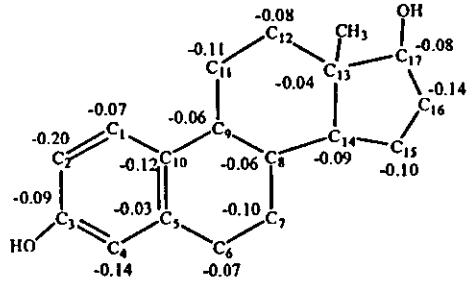

FIGURE 3. Atom partial charge of 17β-estradiol.

ion, and the number of chlorine atoms. While the structures for dichloro-E1 and dichloro-E2 were deduced to be 2,4-dichloro-E1 and 2,4-dichloro-E2 from a chlorine-substitution mechanism, the structure of the monochloro-E1 was speculated to be 2-chloro-E1 and/or 4-chloro-E1. Figure 3 shows the atom partial charges of the E2 molecule. According to a chlorination mechanism, the presence of a negative charge on the phenolic compound will facilitate the chlorination reaction. Thus, the chlorine substitution reaction followed by dehydration will primarily occur at C2 with a partial charge of −0.20, and 2-chloro-E2 will be formed (Figure 3). The 2-chloro-E1 was presumed to be formed by the reaction between 2-chloro-E2 and HOCl. Another possible pathway to form 2-chloro-E1 is for E2 to be oxidized to E1 and then chlorinated to 2-chloro-E1. Experimentally, a trace of E1 was detected after 10 min chlorination. Such a reaction will also occur at C4 with a partial charge of −0.134 to form 4-chloro-E1, although the abundance will be lower than that of 2-chloro-E1 due to the relatively low negative partial charge at C4. Such a reaction mechanism was proposed and reported in our previous paper (*23*).

In addition to these three chlorine-substituted products of E2, four other peaks A−D were observed on the chromatogram, with peak C dominant. Figure 4 shows the mass spectra of the A−D products. The mass spectra of peaks B (20.18 min) and C (20.95 min) show that these two products contain two chlorine atoms with the same molecular ion (M − H)− of *m/z* 355, but with different fragment ions (Figure

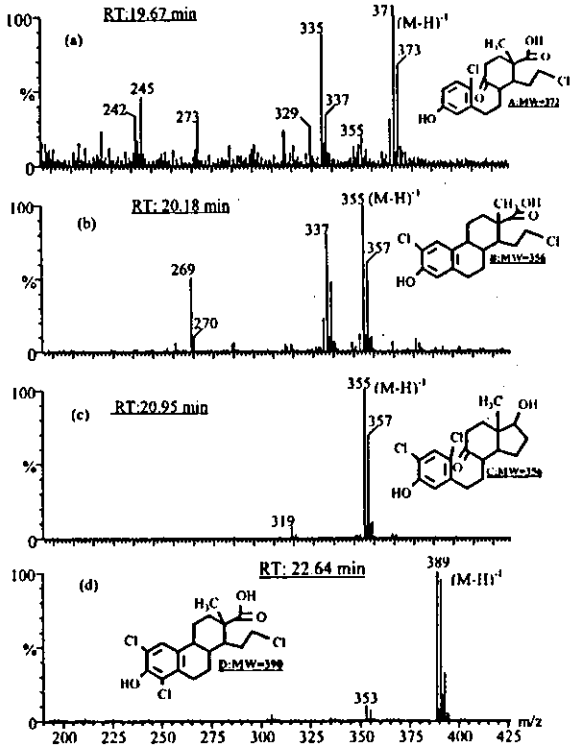

FIGURE 4. LC-MS mass spectra of peaks A−D.

4b,c). From one fragment at *m/z* 319 for peak C and one fragment at *m/z* 337 (two Cl atoms) for product B, it is difficult to deduce their correct structures. An attempt was made, however, to assign structures to products B and C. From Figure 3, it is noted that C10 and C16, having relatively low negative partial charge, are potential reactive sites in addition to C2 and C4. Thus, upon HOCl attacking either carbon atom, two possible structures can be deduced: one resulting from the breakdown between the C9−C10 bond of the above monochloro-E2 and another resulting from the breakdown between C16 and C17 as shown in Figure 4b,c. The molecular ion (M − H)− at *m/z* 355 (two Cl atoms) supports the above possible structure. In the LC-MS method, monitoring of daughter ions produced by in-source CID of analyte ions can provide a valuable confirmation of identity, though with some loss of sensitivity.

To further identify the above two structures, we fractionated the product corresponding to peak C using HPLC, and further analyzed by ESI-LC-MS at a cone voltage of 100 V. Figure 5 shows the spectrum of this fraction. Besides the molecular ion at *m/z* 355 and one fragment at *m/z* 319 which have been found at a cone voltage of 60 V, three other fragments, *m/z* 189, 202, and 227, each containing two chlorine atoms, were obtained. The above structural information supported a breakdown at C10 and C9 and the formation of product C, with the proposed structure as in Figure 4c. Indirectly, the structure of product B was identified as shown in Figure 4b. Considering the structural similarity between E1 and E2, E1 chlorination under experimental conditions similar to those used in E2 chlorination was also conducted. A compound with a molecular ion at *m/z* 353 (two chlorine atoms) and one fragment at *m/z* 317 (one chlorine atom) as shown in Figure 6a was the main product formed in the aqueous chlorination of E1. On the basis of the above structural information from the mass spectrum obtained at a cone voltage of 60 V, only one possible structure (Figure 6a) can be proposed. To obtain more structural information, the fraction corresponding to this product was

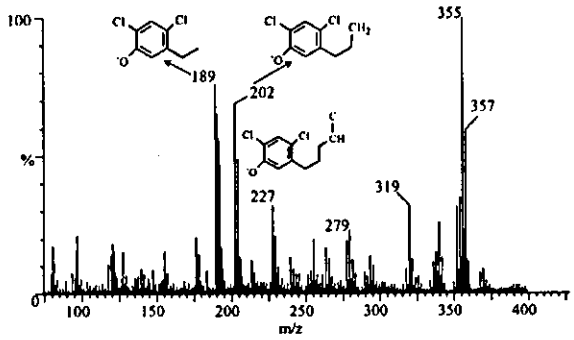

FIGURE 5. LC-ESI-MS mass spectrum of product C: cone voltage, 100 V. Product C (ca. 100 mg/L) was fractionated by HPLC; for other conditions see Materials and Methods.

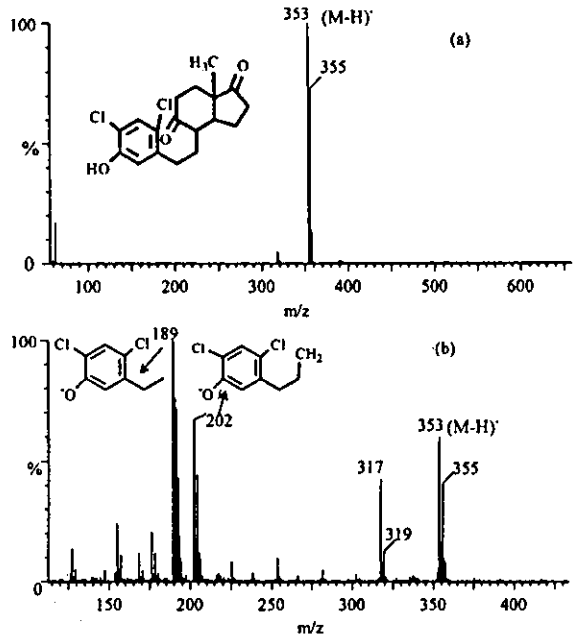

FIGURE 6. LC-ESI-MS mass spectrum of a main product in aqueous chlorinated solution of E1: cone voltage, (a) 50 and (b) 100 V. The product was fractionated by HPLC. For other conditions, see Materials and Methods.

also detected using LC-ESI-MS at a cone voltage of 100 V. The same fragment ions as those of product C (a chlorinated product of E2), *m/z* 189, 202, and 227, were also found in the spectrum of this product of E1 (with molecular ion at *m/z* 353), indicating that the potential of breakdown between C9 and C10 is higher than that between C16 and C17, even for E1.

In addition, the mass spectrum of the compound corresponding to peak D at 22.64 min provided a molecular ion at *m/z* 389 with three Cl atoms (a base ion) and a fragment ion $(M\text{-}Cl\text{-}2H)^-$ at *m/z* 353 as shown in Figure 4d. On the basis of the above structural information, a possible product D, as shown in Figure 4d, was postulated as being formed during the reaction between product C and HOCl. Finally, the mass spectrum of peak A at retention time 19.67 min provided the molecular ion $(M-H)^-$ at *m/z* 371 (two chlorine atoms), and fragment ions at *m/z* 335 with one chlorine atom, and *m/z* 355 with two chlorine atoms. This is indicative of the formation of product A with the structure shown in Figure 4a.

Chlorination Pathways of E2. Figure 7 shows the variation in levels of E2, three chlorine-substituted E2 and E1

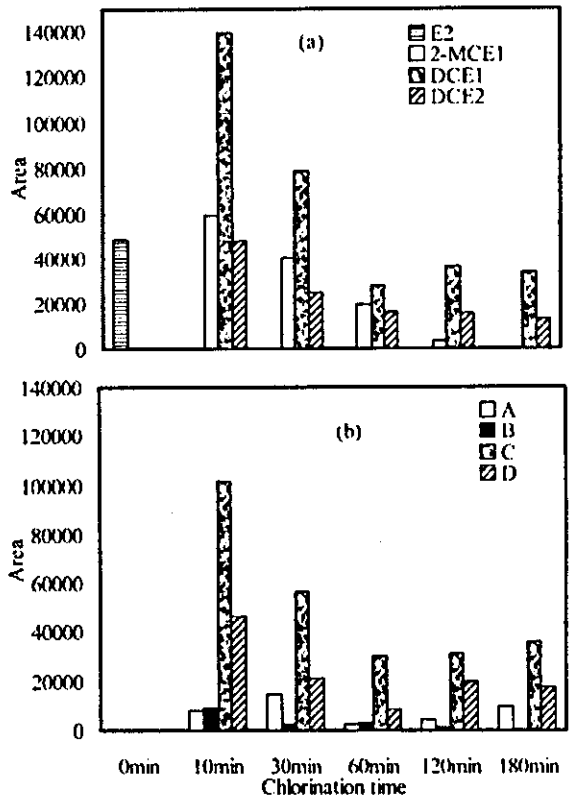

FIGURE 7. Variation of abundances of E2 and its products with chlorination time. E2, 17β-estradiol; 2-MCE1, 2-monochloroestrone; DCE1, dichloroestrone; DCE2, dichloro-17β-estradiol. A–D correspond to peaks A–D in Figure 1.

products, and the other four products from A to D with reaction time. Compound E2 rapidly reacted with HOCl, with almost 100% of E2 having disappeared after 10 min reaction time. Of eight products, the most abundant were 2,4-dichloro-E1, followed by product C with a molecular weight of 356 (assuming similar sensitivity for each product). While monochloro-E1 and B had disappeared after 180 min reaction time, the five other products remained after 180 min when the concentration of sodium hypochlorite was 1.30 mg/L, indicating that these five products are relatively resistant to further reaction with HOCl. On the basis of the above results, pathways for the aqueous chlorination of E2 are proposed, as shown in Figure 8, which includes (1) chlorine substitution reactions followed by dehydration and (2) chlorine substitution reactions followed by cleavage of the C10–C9 bond.

Estrogenic Activity of Aqueous Chlorinated E2 Solution. It was found that many products were formed in the aqueous chlorination of E2. To evaluate the estrogenic activity potentially stemming from E2 in drinking water, it was necessary to investigate the estrogenic activity of these products. A yeast two-hybrid system based on the ligand-dependent interaction of two proteins, a human estrogen receptor and a coactivator, were used to assess the estrogenic potency of the above chlorinated E2 solution, by determining the ligand-induced transcriptional activation. Yeast cells were exposed to chlorinated E2 solutions, and the results for the samples, which were chlorinated for 0, 10, 30, 60, 120, and 180 min, exhibited β-galactosidase activity as shown in Figure 9. While the maximal β-galactosidase activities induced by the chlorinated solution at 10, 30, and 60 min are similar though slightly lower than that before chlorination, the activities of the chlorinated solution at 120 and 180 min are about 40% of that before chlorination. The presence of

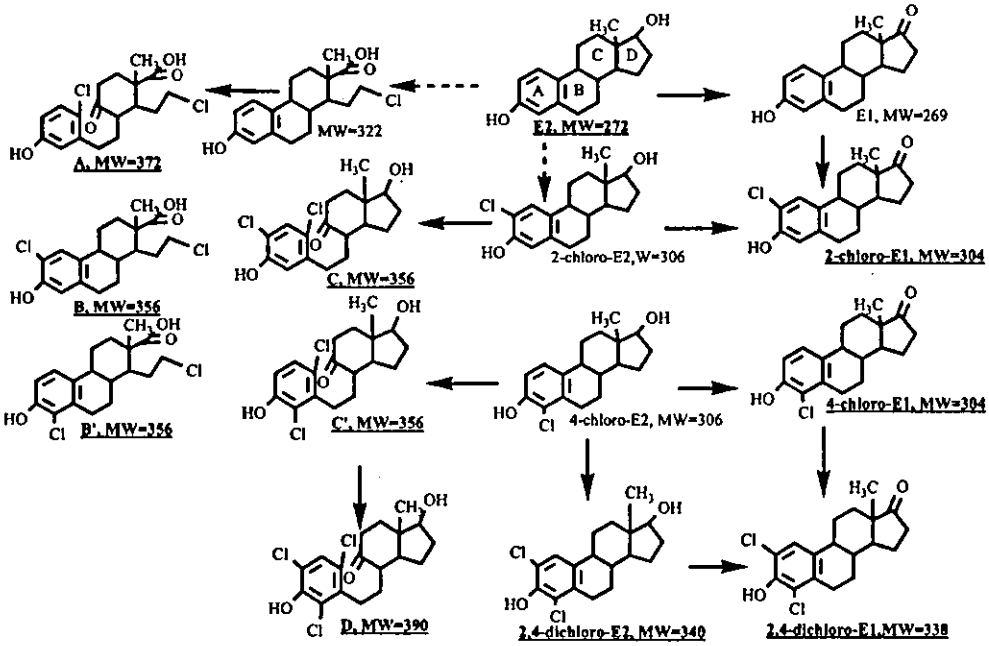

FIGURE 8. Pathways of chloro-substitution reaction between 17β-estradiol and HOCl. Boldface: detected in this experiment; possible reaction even though no product was found.

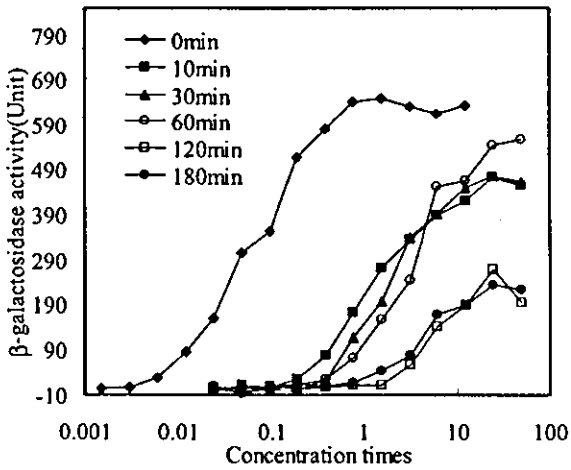

FIGURE 9. Dose–response curve of aqueous chlorinated E2 at various reaction times.

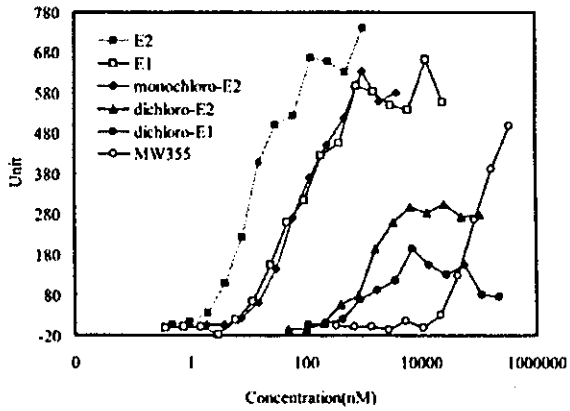

FIGURE 10. Dose–response curves of seven compounds. E1, estrone; E2, 17β-estradiol; monochloro-E2, 2-chloro-E2 and 4-chloro-E2; dichloro-E2, 2,4-dichloro-E2; dichloro-E1, 2,4-dichloro-E1; MW356, product C, a chlorinated product of E2 with molecular weight of 356.

monochloro-E1 in solution for less than 60 min would thus result in a higher β-galactosidase activity than that after 120 min chlorination time. In addition, it is proposed that the solution at 120 min chlorination time elicits maximal β-galactosidase activity similar to that at 180 min chlorination time, because no variation in the kinds of products and their abundances at the two different chlorination times are observed (Figure 7). Besides the difference in β-galactosidase activity, the effective doses showing half-maximal transcriptional response (EC50) occurred at significantly higher dose than before chlorination. The reduction of estrogenic activity of E2 after chlorination based on a vitellogenin induction assay was also reported (*27*).

Finally, to interpret the β-galactosidase activities of the chlorination products in E2 solution, 4-chloro-E2, 2,4-dichloro-E2, and 2,4-dichloro-E1 were synthesized, and product C was fractionated by HPLC. Figure 10 shows the dose–response curves of single 4-chloro-E2, 2,4-dichloro-E2, 2,4-dichloro-E1, and product C together with those of E1 and E2. It was found that 4-chloro-E2 elicited strong estrogenic activity which is almost the same as that of estrone, and the effective concentration showing half-maximal transcriptional response (EC50) is about 10^2 nM. Although monochloro-E2 could not be detected in aqueous chlorinated E2 solution, the presence of 4-chloro-E2 would explain the observed estrogenic activity, from consideration of the structural similarity between E1 and E2. It was found that 2,4-dichloro-E2 elicited weaker β-galactosidase activity than that of monochloro-E2: its maximal β-galactosidase activity was 295 units, which is about 40% that of 4-chloro-E2, and its EC50 is ca. 10^3 nM, which is 10 times higher than that of 4-chloro-E2. From the dose–response curve of 2,4-dichloro-E1, while the maximal activity of 2,4-dichloro-E1 is lower than that of 2,4-dichloro-E2, its EC50 is similar to that of 2,4-dichloro-E2. In addition, product C induced high β-galactosidase activity at the relatively higher concentration of 3.5×10^5 nM. The above results suggest that the estrogenic