Table 3. *mRNA levels of identified proteins in each prostatic lobe in 12-week-old rats*[a]

	PrstC3	CRP1	KS3	SBP	TGase4	IgBPLP	CA2	ESP1	GRP78	PRdx6	Prob	PSP94
VP	233.0 ± 15.0	63.0 ± 5.3	12.1 ± 0.9	3.4 ± 0.1	0	0	0	0	1.5 ± 0.1	0	1.7 ± 0.5	0
LP	52.0 ± 3.4	14.6 ± 1.0	2.9 ± 1.0	1.2 ± 0.1	3.0 ± 1.3	0	8.4 ± 2.0	0	1.2 ± 0.2	0	44.1 ± 16.3	14.5 ± 5.4
DP	7.7 ± 4.6	0.8 ± 0.5	0	0	79.3 ± 2.2	55.7 ± 7.1	7.2 ± 0.7	1.3 ± 0.1	1.8 ± 0.1	1.4 ± 0.3	18.8 ± 2.6	12.3 ± 1.5
AP	2.8 ± 1.1	0.7 ± 0.3	0	0	27.0 ± 7.0	33.4 ± 7.9	2.1 ± 0.2	0	0.8 ± 0.4	0	2.4 ± 0.2	1.2 ± 0.4

Abbreviations: AP, anterior prostate; CA2, carbonic anhydrase II; CRP1, cystatin-related protein 1; DP, dorsal prostate; ESP1, epididymis specific protein 1; GRP78, 78-kDa glucose-regulated protein; IgGBPLP, immunoglobulin G–binding protein-like protein; KS3, kallikrein S3; LP, lateral prostate; PRdx6, preroxiredoxin 6; Prob, probasin; PrstC3, prostatein C3; PSP94, prostate secretory peptide 94; SBP, spermine binding protein; TGase4, transglutaminase 4; VP, ventral prostate.

[a] Mean ± SEM (n = 5). Values are mRNA levels divided by β-actin mRNA levels (mol/mol β-actin). Twelve-week-old male F344 rats were sacrificed. Total RNA was isolated from each prostate lobe and mRNA levels were measured by real-time reverse transcriptase–polymerase chain reaction.

The major secretions in the DP/AP were IgGBPLP and TGase4. IgGBPLP is the predicted protein (AAH99756) derived from the cDNA sequence RGD1311906, which was calculated to contain 1914 amino acids, with a molecular weight of 206 kDa. Because peptide sequencing by the peptide-mass fingerprinting method covered several parts of the entire predicted sequence (17% coverage), the 100-kDa spot probably contains a mixture of cleaved fragments derived from the 206-kDa protein, although this remains to be confirmed. This IgGBPLP seems to be identical to the immunoglobulin G (IgG)-binding protein of 115 kDa reported by Wilhelm et al (2002), who also demonstrated that this protein was actually capable of binding IgG. The role of IgGBPLP may thus be to reduce the immunological responses in the female reproductive tract via IgG binding. TGase4 is a prostate-specific isozyme of transglutaminases (Ca2+-dependent intracellular and extracellular enzymes). TGase4 catalyzes the cross-linking between proteins and/or polyamines, and was first discovered as Factor XIII (Seitz et al, 1990; Ho et al, 1992). In the rat prostate, it had been previously known as dorsal prostatic secretory protein-1 (Wilson and French 1980) and was then identified as a member of the transglutaminase gene family (Ho et al, 1992). In rodents, TGase4 plays a role in producing a stable coagulum by catalyzing the isopeptide cross-link. It is also found expressed in the human prostate. CA2 has been known to be expressed at a very high level in prostate tissue (Fischer and Mawson, 1952) and to be expressed in the epididymal duct epithelium as well. This protein is secreted via the apocrine mode (Wilhelm, 1998). The physiological function of CA2 is linked to the secretion of bicarbonate and proton ions into the seminal fluid (Harkonen et al, 1991).

PRdx6 is a new component of the rat prostatic secretion found in the present study. Because it is an antioxidant enzyme that reduces peroxide and alkyl hydroperoxide to water and alcohol, respectively (Wang et al, 1997), it would presumably act as a seminal plasma antioxidant. GRP78 belongs to the heat shock protein 70 family, which were previously considered to be intercellular proteins. However, a recent proteomic analysis of human prostasomes revealed the presence of heat shock proteins in prostatic secretions (Girotti et al, 1992). In addition, heat shock protein 70 has been reported to be secreted from a variety of prostatic cell lines and to show growth-inhibitory activity (Jones et al, 2004; Wang et al, 2004). GRP78 may have similar activity in the rat prostate. ESP1 is a protein identified as a major epididymis secretory protein. It belongs to the lipocalin superfamily and may be related to cholesterol transport (Girotti et al, 1992). Interestingly,

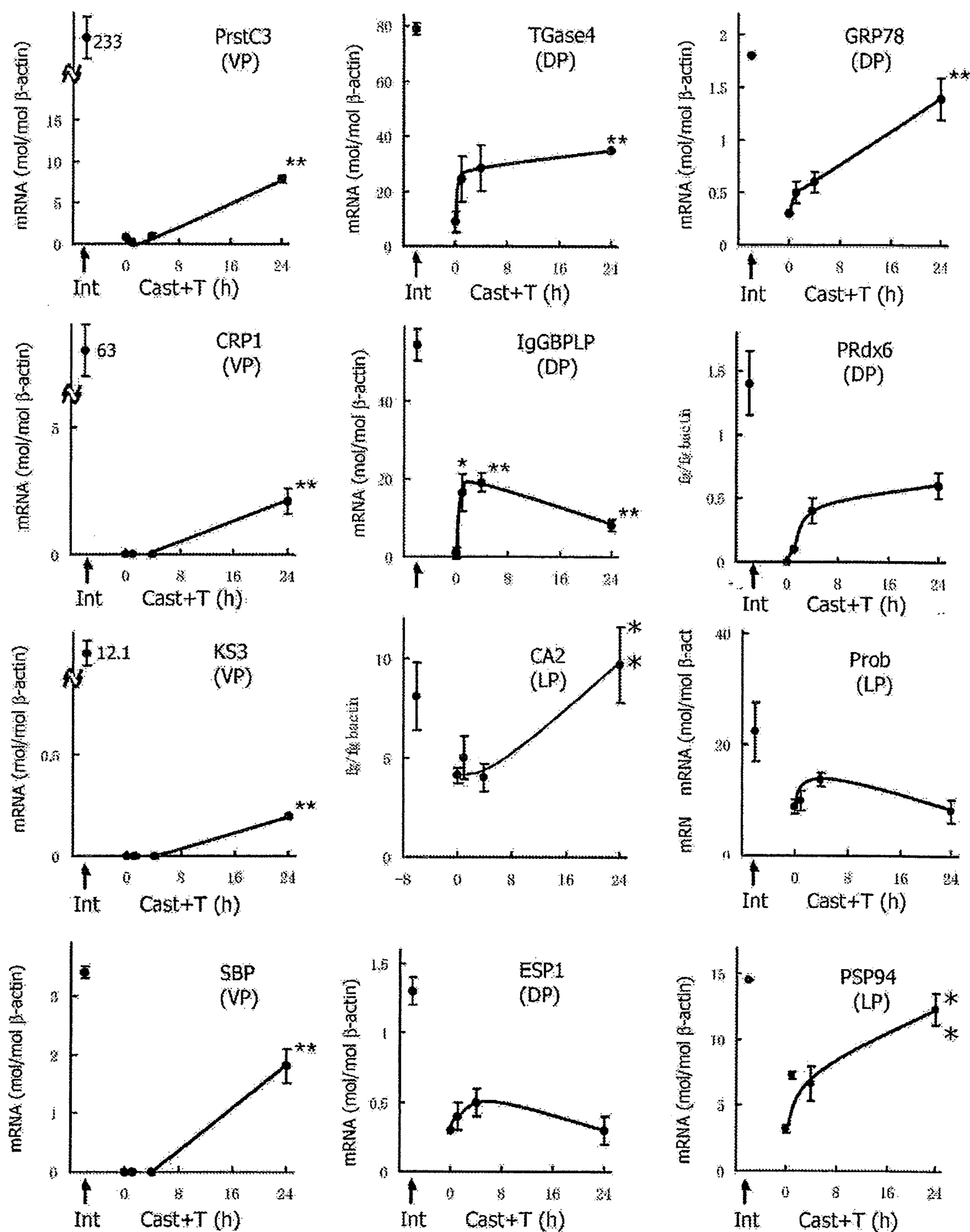

Figure 2. The mRNA levels of the prostatic proteins in the designated prostatic lobes at 0, 1, 4, and 24 hours after a testosterone (T) injection (5 mg/kg body weight, IP) in rats castrated a week previously. Bar indicates mean ± SEM. n = 5. * indicates $P < .05$ vs 0 hours; ** $P < .01$ vs 0 hours.

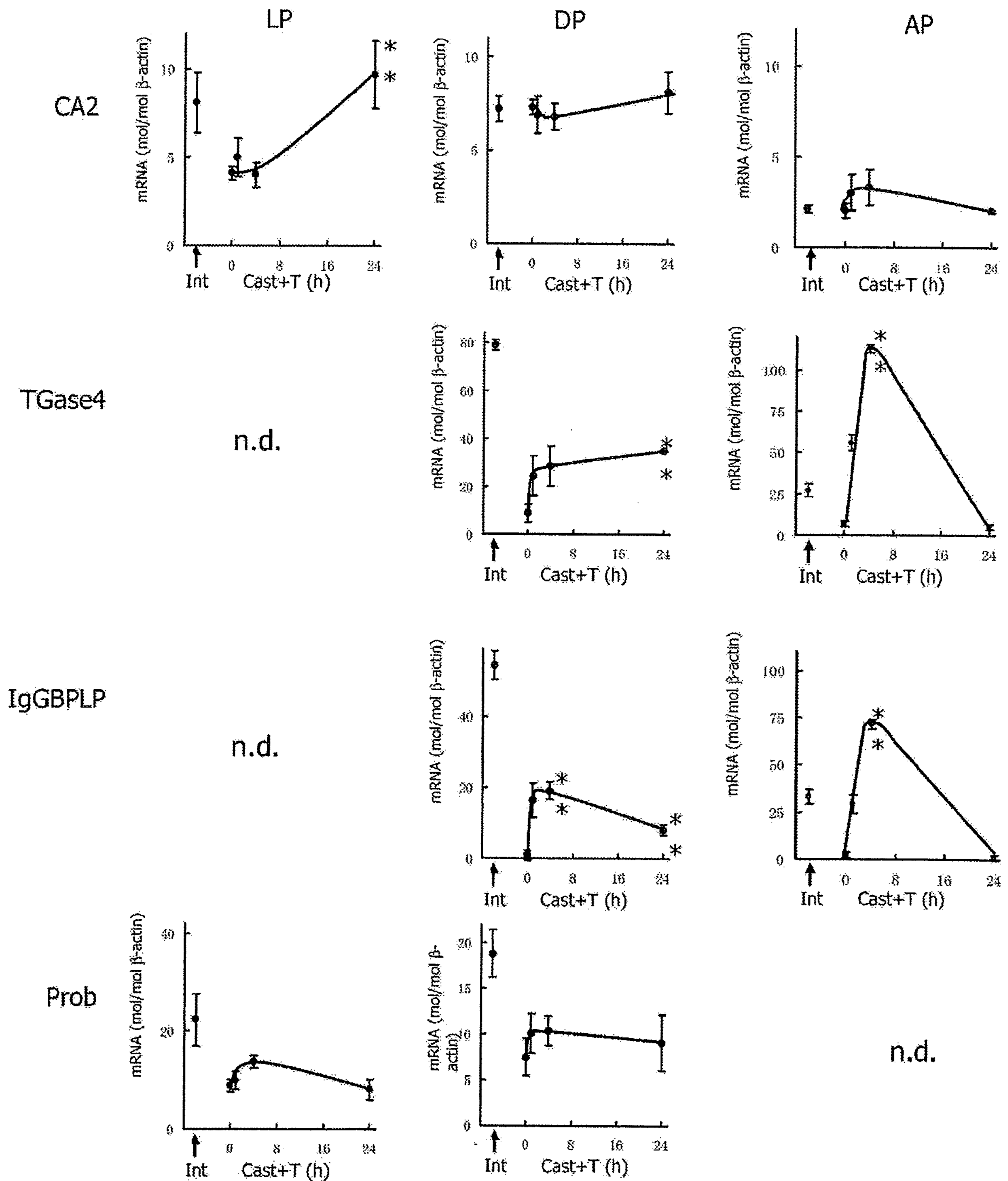

Figure 3. The mRNA levels of the prostatic proteins in the lateral prostate (LP), dorsal prostate (DP), and anterior prostate (AP) at 0, 1, 4, and 24 hours after a testosterone (T) injection (5 mg/kg body weight, IP) in rats castrated a week previously. Bar indicates mean ± SEM. n = 5. ** indicates $P < .05$ vs 0 hours; ** $P < .01$ vs 0 hours; n.d., not detected.

ESP1 turned out to be a gene that causes Niemann-Pick type C disease, a fatal neurodegenerative disorder characterized by a lysosomal accumulation of cholesterol and other lipids within the cells of patients (Naureckiene et al, 2000).

Prostatic proteins are generally regulated by androgen, although the responses differ greatly depending on the proteins and prostatic lobes that are expressed. In a previous study, we demonstrated stricter androgen responses in the VP as compared to the other lobes. For instance, castration decreased probasin mRNA expression to 2% of the control level after a week, while the decrease in the DP was only to 40% (Suzuki et al, 2007), although the genomic upstream regions contain consensus androgen-responsive elements (AREs) that were functional promoters in in vitro reporter assay (Rennie et al, 1993). We examined the androgen-dependent transcriptional regulation of the identified prostate proteins after a single injection of testosterone in castrated animals. The serum testosterone level well exceeded the control level within 1 hour and was still high 24 hours after the injection, as we previously reported (Suzuki et al, 2007). Within 24 hours, most of the mRNA levels had significantly increased, which clearly demonstrated the androgen inducibility of their mRNA levels. In the present study, we found that newly identified IgGBPLP and TGase4 mRNAs were rather strictly regulated by androgen in the DP and the AP. They provide useful models for androgen responsive gene transcription in the DP/AP. The genomic upstream regions of these genes, however, do not contain ARE or ARE-like motifs (Q49907_rat gene in chromosome 1 and tgm4 gene in chromosome 8). Further investigations are required to understand their androgen-responsive mechanisms. The CA2 expression exemplifies the differential androgen dependency of the transcription between lobes, as previously reported (Harkonen et al, 1991), which showed that CA2 mRNA expression was up-regulated by androgen in the LP, whereas it was down-regulated in the DP in SD rats. In the present study, there were comparable responses in CA2 mRNA in the LP but no significant changes in the DP, which may be because of differences in androgen dosages or the mRNA quantification methods. The differential responses of CA2 to androgen suggest the lobe-specific function of this protein. Estrogen and the other hormone may be also involved in the androgen regulation of CA2 (Harkonen et al, 1991).

Recently, proteomic analysis of the human prostate and prostasomes revealed that the human prostate may secrete PRdx6 and GRP78. Another investigation searching for cancer markers found IgG Fc-binding protein expressed in the human prostate (Gazi et al, 2007). The present study shows that IgGBPLP, GRP78, and PRdx6, in addition to PSP94, TGase4, and CA2, are the aspects of secretory functionality common to mice, rats, and humans. The identified secretory proteins should be available as models of androgen-dependent gene regulation and are candidates as markers for prostatic differentiation.

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Affinity for Thyroid Hormone and Estrogen Receptors of Hydroxylated Polybrominated Diphenyl Ethers

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The affinity for thyroid hormone receptor (TR) of polybromodiphenyl ethers (PBDEs) and hydroxylated PBDEs was examined. 4-Hydroxy-2,2′,3,4′,5-pentabromodiphenyl ether (4-OH-BDE-90) and 3-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether (3-OH-BDE-47) markedly inhibited the binding of triiodothyronine (1×10^{-10} M) to TR in the concentration range of 1×10^{-6}–1×10^{-4} M. 2,3,4,5,6-Pentabromophenol (PBP) also showed an inhibitory effect at 1×10^{-5}–1×10^{-4} M. However, 2,2′,3,4,4′,5′-hexabromodiphenyl ether (BDE-138), decabromodiphenyl ether (DBDE), 4-methoxy-2,2′,3,4′,5-pentabromodiphenyl ether (4-MeO-BDE-90), 4′-hydroxy-2,2′,4,5′-tetrabromodiphenyl ether (4′-OH-BDE-49), 4-hydroxy-2,2′,3,4′-tetrabromodiphenyl ether (4-OH-BDE-42), 4′-hydroxy-2,2′,4-tribromodiphenyl ether (4′-OH-BDE-17), 3′-hydroxy-2,4-dibromodiphenyl ether (3′-OH-BDE-7), 2,4,6-tribromophenol (TBP) and tetrabromohydroquinone (TBHQ) did not show affinity for TR. In contrast, 4′-OH-BDE-17 and 3′-OH-BDE-7 exhibited estrogenic activity in estrogen-responsive reporter assay using MCF-7 cells at the concentration of 1×10^{-5} M. However, adjacent bromo substitution of 3- or 4-hydroxylated PBDEs markedly decreased the estrogenic activity. These results suggest that hydroxylated PBDEs act as thyroid hormone-like agents, as well as estrogens, that a 4- or 3-hydroxyl group in PBDEs is essential for thyroid hormonal and estrogenic activities, and that adjacent dibromo substitution favors thyroid hormonal activity, but not estrogenic activity.

Key words——polybrominated diphenyl ether, thyroid hormonal activity, estrogenic activity, hydroxylated polybrominated diphenyl ether

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INTRODUCTION

Brominated flame retardants are widely used throughout the world in electronic circuit boards and other electronic equipment.[1–5] Polybrominated diphenyl ethers (PBDEs) and tetrabromobisphenol A (TBBPA) have been detected in environmental samples such as sediments, fish, birds, and also in human breast milk and blood.[6–14] Exposure to PBDEs can lead to thyroid hormone disturbances and neurotoxicity in wildlife and humans.[15–19]

It has been reported that some PBDEs and TBBPA have estrogenic, anti-androgenic and anti-progesteronic activities *in vitro*.[19–22] Ceccatelli *et al.* reported that a PBDE, PBDE 99, can cause disruption of estrogenic action *in vivo* in rats.[23] Richardson *et al.* reported that BDE 47 caused disruption of thyroid hormonal action in mice *in vivo*.[24] Lilienthal *et al.* also reported an effect of PBDE 99 on sexual development and sexually dimorphic behavior in rats.[25] Several PBDEs have been reported to cause thyroid hormone disruption.[26–28] It has been suggested that hydroxylated PBDEs may disrupt thyroid hormone status, because of their structural similarity to thyroid hormone. Hydroxylated PBDEs may have high binding affinity for the serum thyroid hormone binding protein transthyretin (TTR), thyroid hormone receptor (TR), thereby displacing the natural ligand. However, the exact mechanisms of interference with thyroid hormonal action are not fully understood.

In this report, we tested with the thyroid hormonal and estrogenic activities of PBDEs, including their hydroxylated derivatives, as determined by means of binding assay with TR from the rat pituitary cell line MtT/E-2 and estrogen-

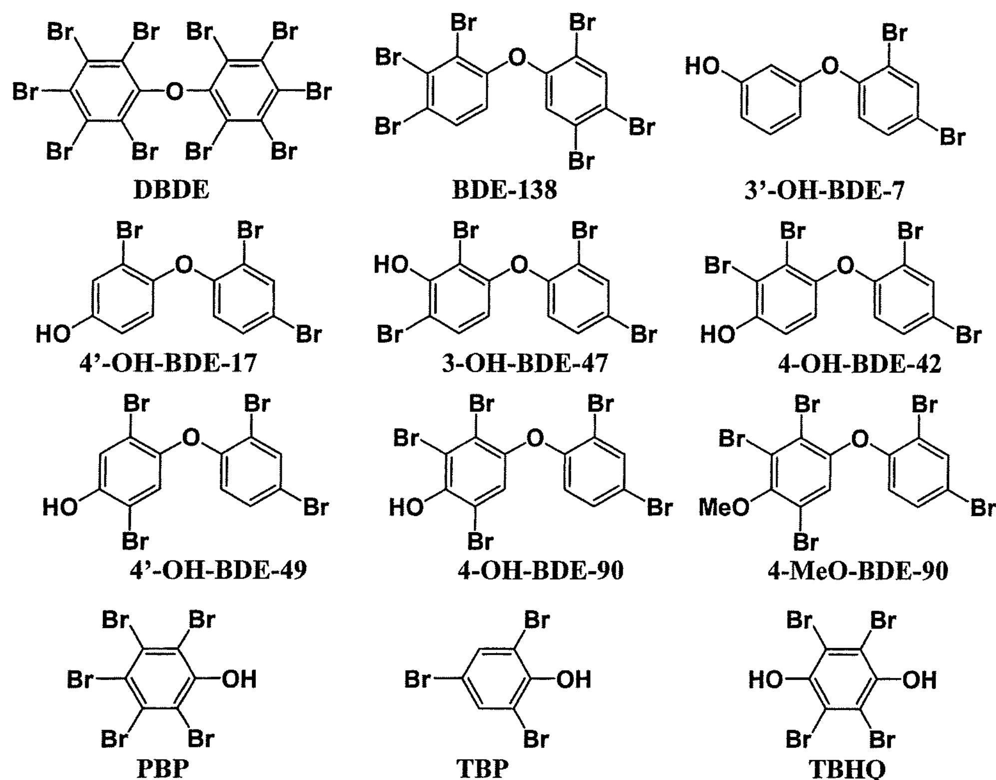

Fig. 1. Structures of PBDEs and Hydroxylated PBDEs Used in this Study

responsive element (ERE)-luciferase reporter assay using MCF-7 cells. As substrates, we used 12 PBDEs and related compounds, 2,2′,3,4,4′,5′-hexabromodiphenyl ether (BDE-138), decabromodiphenyl ether (DBDE), 4-hydroxy-2,2′,3,4′-tetrabromodiphenyl ether (4-OH-BDE-42), 4′-hydroxy-2,2′,4-tribromodiphenyl ether (4′-OH-BDE-17), 4-hydroxy-2,2′,3,4′,5-pentabromodiphenyl ether (4-OH-BDE-90), 3-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether (3-OH-BDE-47), 4′-hydroxy-2,2′,4,5′-tetrabromodiphenyl ether (4′-OH-BDE-49), 3′-hydroxy-2,4-dibromodiphenyl ether (3′-OH-BDE-7), 4-methoxy-2,2′,3,4′,5-pentabromodiphenyl ether (4-MeO-BDE-90), 2,3,4,5,6-pentabromophenol (PBP), 2,4,6-tribromophenol (TBP) and tetrabromohydroquinone (TBHQ) (Fig. 1). We found that hydroxylated PBDEs exhibit significant thyroid hormonal activity, as well as estrogenic activity.

MATERIALS AND METHODS

Chemicals—— ^{125}I-T3 (3,5,3′-^{125}I, radiochemical purity > 95%, 28.8 TBq/mmol) was purchased from NEN Life Science Products (Boston, MA, U.S.A.). Hydroxylated PBDEs and other PBDEs were obtained from Accu Standard (New Haven, CT, U.S.A.), and 17β-estradiol (E2) was from Sigma (St. Louis, MO, U.S.A.). Test compounds were solubilized in dimethyl sulfoxide, and the assay was conducted in 0.1% dimethyl sulfoxide solution. Cells of a human breast cancer cell line, MCF-7, were obtained from the Health Science Research Resources Bank (Osaka, Japan). The rat pituitary cell line, MtT/E-2, was established in our laboratory.[29)]

Competitive Binding Assay to TR—— Nuclear extracts of MtT/E were used for the assay, since this cell line expresses large amounts of TR. MtT/E-2 cells were homogenized in 0.32 M sucrose solution containing 3 mM $MgCl_2$ and 1 mM dithiothreitol, and centrifuged at 700 g for 10 min. The pellets were resuspended in 2.4 M sucrose with $MgCl_2$ and centrifuged at 53000 g for 45 min. The resulting nuclear pellets were resuspended in TMDS buffer (2 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 0.32 M sucrose, pH 7.4). Various concentrations of test chemicals and 3 nM ^{125}I-T3 were incubated in 0.2 ml of the nuclear suspension at 37°C for 40 min. After incubation, 0.25 ml of 2% Triton X-100 was added to terminate the reaction, and the mixture

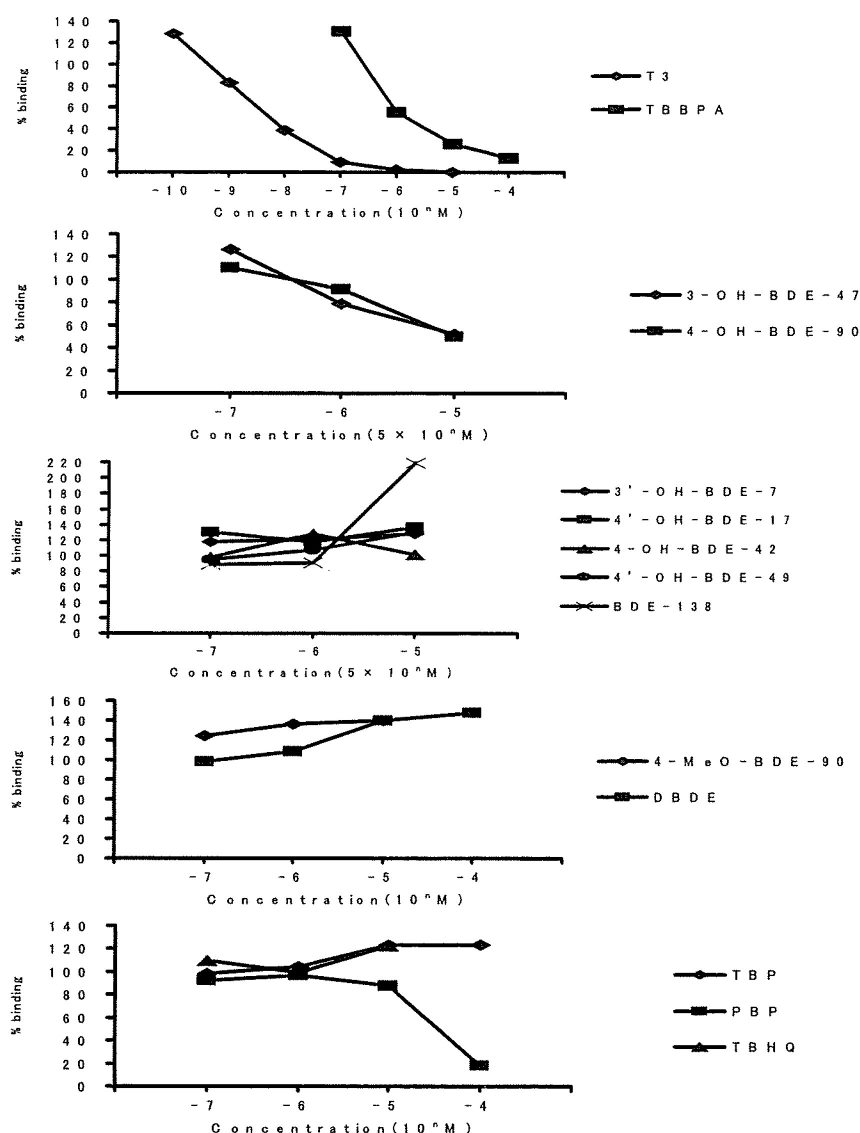

Fig. 2. Binding Assay of Test Compounds to Thyroid Hormone Receptor

Each value represents the mean of triplicate determinations. Activity was expressed relative to the control with no added test compound. T3; L-3,5,3′-triiodothyronine, TBBPA; tetrabromobisphenol A, 3-OH-BDE-47; 3-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether, 4-OH-BDE-90; 4-hydroxy-2,2′,3,4′,5-pentabromodiphenyl ether, 3′-OH-BDE-7; 3′-hydroxy-2,4-dibromodiphenyl ether, 4′-OH-BDE-17; 4′-hydroxy-2,2′,4-tribromodiphenyl ether, 4-OH-BDE-42; 4-hydroxy-2,2′,3,4′-tetrabromodiphenyl ether, 4′-OH-BDE-49; 4′-hydroxy-2,2′,4,5′-tetrabromodiphenyl ether, BDE-138; 2,2′,3,4,4′,5′-hexabromodiphenyl ether, 4-MeO-BDE-90; 4-methoxy-2,2′,3,4′,5-pentabromodiphenyl ether, DBDE; decabromodiphenyl ether, TBP; 2,4,6-tribromophenol, PBP; 2,3,4,5,6-pentabromophenol, TBHQ; tetrabromohydroquinone.

was centrifuged at 1000 *g* for 10 min. The pellets were washed 2 times with 1 ml of TMDS buffer and the supernatant was removed. Radioactivity of the pellets was counted with a gamma counter (Wallac Wizard 1480, Perkin Elmer Life Sciences, Boston, MA, U.S.A.).

Assay of Estrogenic Activity of Hydroxylated PBDEs and Related Compounds—— ERE-luciferase reporter assay using MCF-7 cells was performed according to the previously reported method.[30]

RESULTS

Competitive Binding Assay for TR of Hyydroxylated PBDEs and Related Compounds

The inhibitory effects of PBDEs and hydroxylated PBDEs on binding of T3 to TR were examined. T3 competitively inhibited the binding of ^{125}I-T3 (1×10^{-10} M) to TR in the range of 1×10^{-9}–1×10^{-6} M, and TBBPA also showed an inhibitory effect at 10^{-6}–10^{-4} M. 4-OH-BDE-90 and 3-OH-BDE-47 also markedly inhibited the binding of

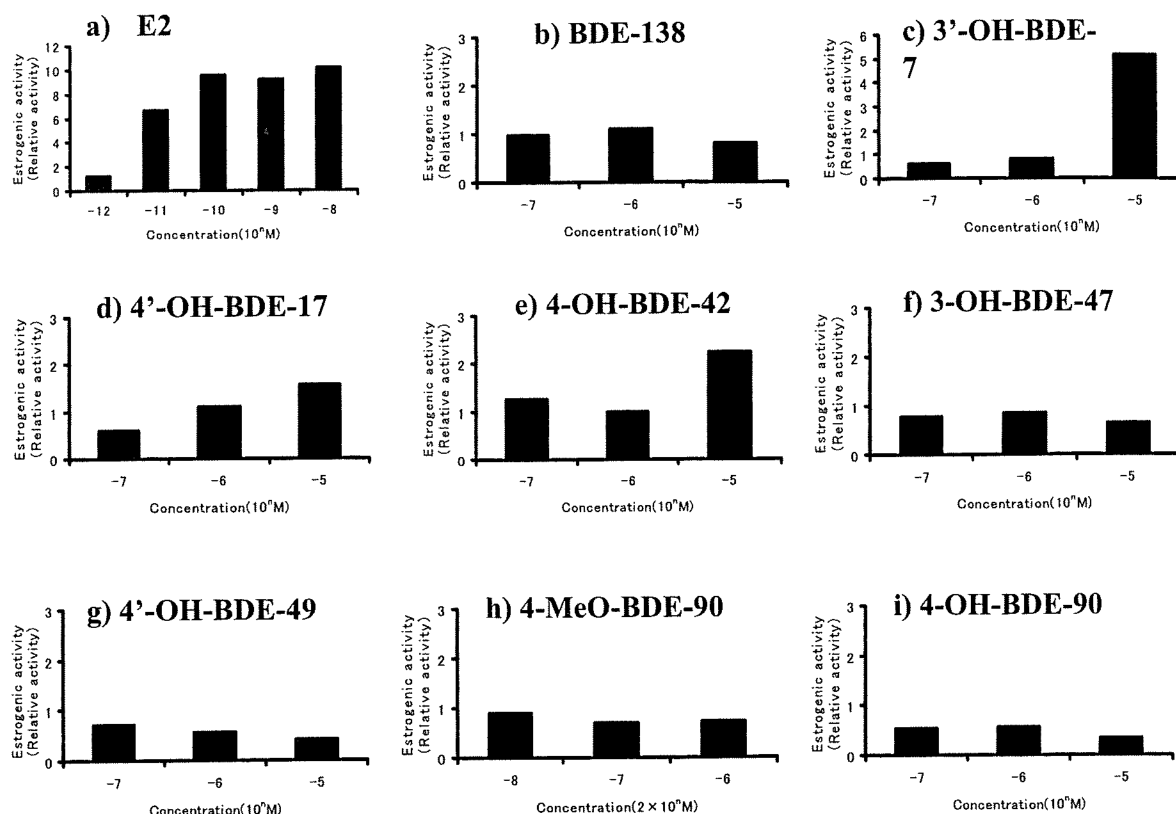

Fig. 3. Estrogenic Activity of PBDEs and Related Compounds in ERE-luciferase Reporter Assay Using MCF-7 Cells

Each value represents the mean of triplicate determinations. Estrogenic activity of PBDEs was expressed as relative activity with respect to the control using MCF-7 cells. E2; 17β-estradiol, BDE-138; 2,2′,3,4,4′,5′-hexabromodiphenyl ether, 3′-OH-BDE-7; 3′-hydroxy-2,4-dibromodiphenyl ether, 4′-OH-BDE-17; 4′-hydroxy-2,2′,4-tribromodiphenyl ether, 4-OH-BDE-42; 4-hydroxy-2,2′,3,4′-tetrabromodiphenyl ether, 3-OH-BDE-47; 3-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether, 4′-OH-BDE-49; 4′-hydroxy-2,2′,4,5′-tetrabromodiphenyl ether, 4-MeO-BDE-90; 4-methoxy-2,2′,3,4′,5-pentabromodiphenyl ether, 4-OH-BDE-90; 4-hydroxy-2,2′,3,4′,5-pentabromodiphenyl ether.

^{125}I-T3 to the receptor in the concentration range of 1×10^{-6}–1×10^{-4} M. PBP showed an inhibitory effect at 1×10^{-5}–1×10^{-4} M. BDE-138, DBDE, 4-MeO-BDE-90, 4′-OH-BDE-49, 4-OH-BDE-42, 4′-OH-BDE-17, 3′-OH-BDE-7, TBP and TBHQ showed little or no affinity. Elevated binding affinity was observed for hydroxylated PBDEs with bromine substitution at both adjacent positions to the hydroxyl group. 4-Hydroxy-PBDEs brominated at the 3- or 5-position, but not both, showed little activity. Hydroxylated PBDEs with no adjacent bromine substitution of the phenyl ring, and 4-methoxy-PBDEs also showed little affinity (Fig. 2).

Estrogenic Activity of PBDEs and Hydroxylated PBDEs

4′-OH-BDE-17 and 3′-OH-BDE-7 exhibited estrogenic activity in estrogen-responsive reporter assay using MCF-7 cells in the concentration range of 1×10^{-6}–1×10^{-5} M. However, no estrogenic activity of other 4-hydroxy-PBDEs or PBDEs was observed. These experiments indicate that a hydroxyl group of PBDEs is essential for estrogenic activity, but 3- or 4-hydroxyl PBDEs with ortho-bromine substituents show decreased estrogenic activity (Fig. 3).

DISCUSSION

In this study, the thyroid hormonal potency of PBDEs was examined, in view of their structural resemblance to the thyroid hormones. We found that hydroxylated PBDEs show thyroid hormone-disrupting activity through interaction with TR, though PBDEs do not. Recently, thyroid hormone-disrupting action of some PBDEs and hydroxy-PBDEs has been discussed.[31] Meerts *et al.* reported that some PBDEs exhibited binding capability to TTR after metabolic conversion by rat liver microsomes.[32] They suggested that the hydroxylated PBDEs thus formed exhibited the binding ac-

tivity with TTR. Hallgren and Danerud also suggested that hydroxylated metabolites of 2,2′,4,4′-tetrabromodiphenyl ether bind TTR in rats *in vivo*.[15] Schriks *et al.* reported that synthetic T2, T3 and T4-like PBDEs show thyroid hormone-like activity.[33] Dietrich *et al.* reported that some brominated thyroxine analogues have thyroid hormonal activity.[34] Hamers *et al.* reported that some PBDEs disrupt the endocrine system by binding to TTR, and exhibit progesterone receptor (PR)- and androgen receptor (AR)-antagonistic effects in the presence of T3.[19] We present here direct evidence that some hydroxylated PBDEs bind to TR.

We found that PBDEs substituted with two bromo atoms adjacent to the hydroxyl group on an aromatic ring show thyroid hormonal activity, though other PBDEs do not. A 3- or 4-hydroxyl group and two adjacent bromo substituents on the phenyl group seem to be essential structural factors for binding to TR. In this respect, the compounds apparently resemble T4, rather than T3. However, the atomic size of bromine is much smaller than that of iodine. In view of the affinity of hydroxylated PBDEs for TR, two adjacent bromo substituents may be favorable for binding to TR. We also recognized a similar structural requirement for TR affinity in a brominated bisphenol A, TBBPA, and also chlorinated and methylated bisphenol A.[20] 3,5-Substituents of bisphenol A are also necessary for thyroid hormonal activity. Another brominated phenyl ring substituted at the 1-position of the phenyl ring bearing the 3- or 4-hydroxyl group also seems to be necessary for high binding affinity for TR, because TBHQ and TBP showed weaker activity. Hydroxy-polychlorinated biphenyls (PCBs) were also reported to show both estrogenic and thyroid hormone-disrupting activities.[35–38] Iwasaki *et al.* reported that 4-hydroxy-2′,3,3′,4′,5′-pentachlorobiphenyl acts as an antagonist by suppressing the interaction of TR and a coactivator.[39] We also reported that a 4-hydroxyl group and adjacent 3,5-dichloro substituents on the phenyl group seem to be essential structural factors for binding to TR, in agreement with the present findings.[35] In contrast, we found that 4-OH-2′,4′,6′-trichlorobiphenyl, a 4-hydroxy-PCB without 3,5-chlorine atoms, is estrogenic in an estrogen-responsive reporter test using human breast cancer cell line MCF-7. However, 4-hydroxy-3,5-dichlorinated biphenyl exhibited little estrogenic activity.[35] These results are also consistent with the estrogenic activity of hydroxylated PBDEs found in this study.

There are other mechanisms through which environmental contaminants may interact with the thyroid hormone system. These include direct toxicity at the thyroid gland, which can lead to decreased synthesis of thyroid hormones, disturbance of thyroid hormone metabolism, and interaction with thyroid hormone transport proteins.[40–44] Richardson *et al.* demonstrated the induction of glucuronidation and thyroid hormone transporter as mechanisms leading to a decreased level of thyroid hormone after administration of BDE 47 to mice.[24] PBDE-99 reduced the thyroxin level in rat dames after a single gestational exposure, and also reduced hepatic enzyme activity.[45] Hydroxylated PBDEs bind to human TTR and thyroid-binding globulin *in vitro*.[32] In the current study, it was shown that hydroxylated PBDEs interact with TR. These results suggest that hydroxylated PBDEs have the potential to disrupt thyroid hormonal activity *in vivo* by interaction with TR, besides binding with TTR. Further work is necessary to assess the *in vivo* endocrine-disrupting action of hydroxylated PBDEs, taking into account the other thyroid hormone-disrupting actions of these compounds. Thyroid hormonal and estrogenic activities of hydroxylated PBDEs observed *in vitro* may reflect endocrinal toxicity *in vivo*. We demonstrated that amphibian metamorphosis is suppressed by TBBPA and related compounds.[46,47]

In conclusion, the structural requirements of hydroxylated PBDEs for thyroid hormonal activity are a 3- or 4-hydroxyl group and two adjacent bromine substitutions adjacent to the hydroxyl group. The requirement for estrogenic activity is a 3- or 4-hydroxyl group, but adjacent bromine substitution of hydroxy-PBDEs reduces the estrogenic activity.

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— Review —

Metabolic Activation of Proestrogens in the Environment by Cytochrome P450 System

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Liver microsome-mediated activation of proestrogens in the environment is reviewed here. Proestrogens such as methoxychlor, *trans*-stilbene, diphenyl, diphenylmethane, 2,2-diphenylpropane, benzo[*a*]pyrene, benzophenone, 2-nitrofluorene (NF), chalcone, *trans*-4-phenyl-3-buten-2-one and styrene oligomers are negative in *in vitro* estrogen screening tests. However, those proestrogens exhibit estrogenic activity after metabolic activation by the microsomal cytochrome P450 system. In these cases, hydroxylated derivatives of the compounds are formed as major metabolites, and these metabolites exhibit significant estrogenic activities. Thus, the estrogenic activities of proestrogenic compounds are a consequence of metabolism of the parent compounds. Various candidates for proestrogens among medicines and insecticides are also discussed.

Key words —— estrogenic activity, proestrogen, metabolic activation, cytochrome P450, *trans*-stilbene, diphenyl, styrene oligomer

INTRODUCTION

Various man-made chemicals mimic the biological activity of hormones such as sex hormones and thyroid hormone, thereby interfering with hormone receptor function. These chemicals are called endocrine disrupters, and include various persistent chlorinated pesticides, such as 1,1,1-trichloro-2,2-bis(2-chlorophenyl-4-chlorophenyl)ethane (*o,p′*-DDT), dieldrin, kepone, methoxychlor and some polychlorinated biphenyl congeners, and industrial chemicals such as the plasticizer bisphenol A, the surfactant breakdown product nonylphenol and some polychlorinated biphenyl congeners.[1,2] Quantitative structure-activity relationship (QSAR) studies on the structural features of estrogen receptor ligands show that an unhindered hydroxyl group on an aryl ring and a hydrophobic group attached *para* to the hydroxyl group are essential.[3–6] Ligand binding assay and studies in a reporter/transcriptional system for the estrogen receptor support the requirement for these structural features.[7,8] Among such compounds, those which are lipophilic and persistent may be accumulated through the food web, posing a health threat to humans and animals. Endogenous estrogens have been shown to have multiple sites of activity and to exert biological effects. Many so-called xenoestrogens produce a wide variety of toxic effects in animals.

Xenoestrogens can accumulate in our environment, and may play a role in the increasing incidence of breast cancer, testicular cancer, and other problems of the reproductive system in humans. It is therefore important to screen environmental contaminants for estrogenic activity. Their metabolites also need to be identified and screened in order to identify proestrogens, which are activated to estrogens by metabolic systems. Several reports indicate that proestrogens, which act as xenoestrogens after metabolic activation, exist in the environment.[9–16] The potential of these proestrogens, such as methoxychlor, for endocrine disruption needs to be assessed.

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In this review, we cover environmental compounds that are not directly estrogenic, but which are activated by the liver microsomal metabolic system in the mammalian body after ingestion. These include methoxychlor, *trans*-stilbene, diphenyl, diphenylmethane, polycyclic aromatic hydrocarbons, nitropolycyclic aromatic hydrocarbons, α,β-unsatulated ketone, styrene oligomers and others.

ACTIVATION OF *TRANS*-STILBENE AND RELATED COMPOUNDS BY THE CYTOCHROME P450 SYSTEM

It is well known that *trans*-stilbene derivatives, such as diethylstilbestrol (DES), euvestin, 4,4′-diaminostilbene and pinosylvin, have a variety of biological actions, including hormonal, hypocholesterolemic, sympathomimetic, antifungal, antibacterial, antimalarial and anticancer activities.[17–19] DES, a potential estrogen, has been used medically as a substitute for endogenous estrogen and as a hormonal therapy for prostate or breast cancer, and also to prevent threatened abortions.[20,21] However, it may induce vaginal adenocarcinoma.[22] Its estrogenic activity is similar to that of β-estradiol in *in vitro* estrogen screening tests. However, the estrogenic activity of *trans*-stilbene, which is the parent compound of stilbene derivatives and is used as an industrial material, has not been extensively examined.

trans-Stilbene is not estrogenic in the estrogen screening tests. However, when *trans*-stilbene was incubated with liver microsomes of 3-methylcholanthrene-treated rats in the presence of NADPH, the extract of the incubation mixture exhibited an estrogenic effect in the concentration range of 10^{-5}–10^{-6} M. In contrast, *cis*-stilbene showed little estrogenic activity after incubation with liver microsomes.[9] When *trans*-stilbene was incubated with liver microsomal enzyme system of 3-methylcholanthrene-treated rats, *trans*-4-hydroxystilbene and *trans*-4,4′-dihydroxystilbene were both formed, though *trans*-4-hydroxystilbene was predominant. Human cytochrome P450 1A1 and 1A2 isoforms expressed in human lymphoblastoid cells catalyzed both oxidations.[23] In the case of *cis*-stilbene, such hydroxylated metabolites were not detected (Fig. 1).

trans-4-Hydroxystilbene and *trans*-4,4′-dihydroxystilbene both showed estrogenic activity similar to that of DES in estrogen screening tests. Thus, *trans*-stilbene was converted to the active metabolites, *trans*-4-hydroxystilbene and *trans*-4,4′-dihydroxystilbene, by rat liver microsomes, and so the estrogenic activity of *trans*-stilbene might be due mainly to *trans*-4-hydroxystilbene, with some contribution from *trans*-4,4′-dihydroxystilbene. In contrast, *cis*-stilbene was not metabolized to the corresponding hydroxylated metabolites with liver microsomes (Fig. 1). In an *in vivo* estrogenicity test using ovariectomized (OVX) rats, *trans*-stilbene was positive, as well as 4-hydroxylated stilbene.[24] This shows that *trans*-stilbene exhibited estrogenic activity after metabolic activation *in vivo*.

It was demonstrated that resveratrol, which is a derivative of stilbene found in grapes and wine, is an agonist for the estrogen receptor.[25] Among stilbene-related compounds, metabolic activation of *trans*-stilbene oxide, *trans*-α-methylstilbene, tolan, dibenzyl and azobenzene to estrogenic compounds by the cytochrome P450 system was also demonstrated. These compounds did not show

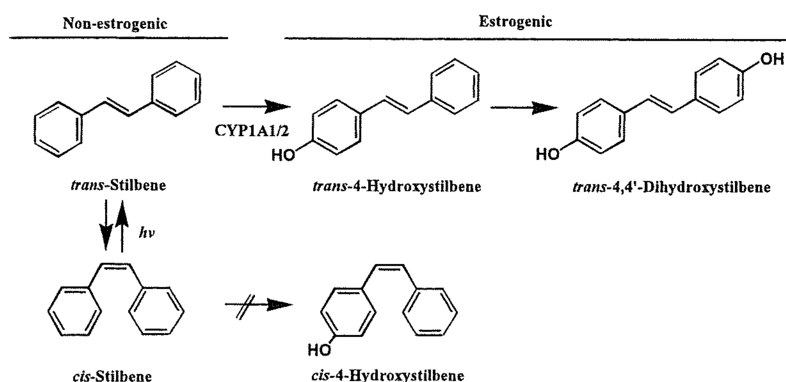

Fig. 1. Metabolic Pathways for the Activation of *trans*-Stilbene to Estrogens by the Cytochrome P450 System

estrogenic activity, but exhibited estrogenic activity after metabolic activation by liver microsomes from 3-methylcholanthrene- or phenobarbital-treated rats (Fig. 2).[26] Furthermore, the estrogenic activities of several stilbene derivatives were compared. The 4-hydroxyl group of the A-ring plays the most important role, but nitro and amino substituents also result in some estrogenic activity. The vinyl linkage is necessary for high activity. The hydrophobic B-ring plays an important role, because the estrogenic activity of hydroxystilbene is higher than that of hydroxystyrene. The structural requirements for the estrogenic activities of stilbene derivatives were proposed to be as shown in Fig. 3. A *p*-hydroxy group in the A-phenyl ring, vinyl linkage, a B-phenyl ring and hydrophobicity of the linkage are necessary for the maximal activity of stilbene derivatives.[26]

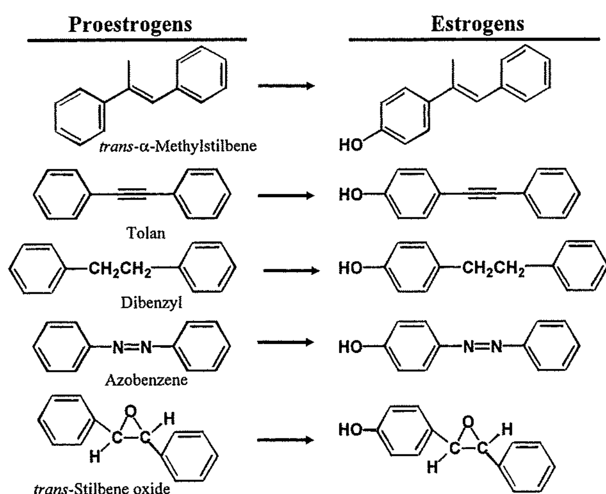

Fig. 2. Metabolic Activation of Stilbene-related Compounds to Estrogens

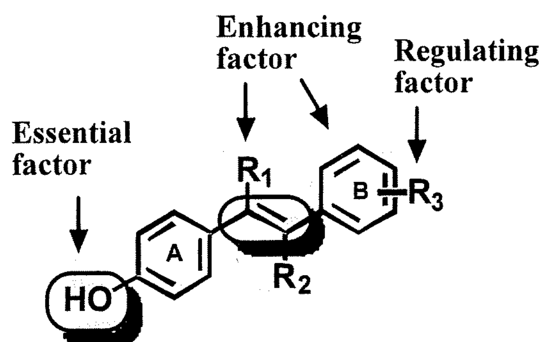

Fig. 3. Structural Requirement of Stilbene Derivatives for Estrogenic Activity

ACTIVATION OF DIPHENYL AND RELATED COMPOUNDS BY THE CYP1A1/2 AND 2B1 SYSTEMS

Diphenyl is used as an antifungal agent for citrus fruits, and also as wrapping paper for impregnated fruit. Under improper conditions, it has been toxic to production workers.[27,28] Diphenylmethane is also used as a dye carrier and synthetic intermediate, similarly to diphenyl. Diphenyl is metabolized to hydroxylated diphenyls. 4-Hydroxydiphenyl, 4,4′-dihydroxydiphenyl and 3,4-dihydroxydiphenyl were identified as urinary metabolites of diphenyl in experimental animals.[29] 2-Hydroxydiphenyl, 2,4′-dihydroxydiphenyl and 3-hydroxydiphenyl were also identified as *in vitro* metabolites with liver microsomes of various animals.[30] These phenylphenols are used as household insecticides, especially for indoor applications,[31] and as intermediates in the manufacture of rubber and resins.

Diphenyl, diphenylmethane and 2,2-diphenylpropane were negative in estrogen screening tests. However, they exhibited estrogenic activity after incubation with liver microsomes of 3-methylcholanthrene-treated rats in the cases of diphenyl and diphenylmethane, or after incubation with liver microsomes of phenobarbital-treated rats in the cases of diphenyl and 2,2-diphenylpropane.[10] When diphenyl was incubated with liver microsomes of phenobarbital- and 3-methylcholanthrene-treated rats in the presence of NADPH for the detection of the estrogenic metabolites, four metabolites (2-hydroxydiphenyl, 3-hydroxydiphenyl, 4-hydroxydiphenyl and 4,4′-dihydroxydiphenyl) were detected. 4,4′-Dihydroxydiphenylmethane and 4-hydroxydiphenylmethane were also detected as metabolites of diphenylmethane with liver microsomes of 3-methylcholanthrene-treated rats. Bisphenol A [2,2-bis(4-hydroxyphenyl)propane] and 2-(4-hydroxyphenyl)-2-phenylpropane were detected as metabolites of 2,2-diphenylpropane with liver microsomes of phenobarbital-treated rats. The estrogenic activity of bisphenol A, which is an active metabolite of 2,2-diphenylpropane in this case, is well-known. However, the amounts of monohydroxyl derivatives of these compounds were much higher than those of the 4,4′-dihydroxyl derivatives (bisphenol A).

These hydroxylated derivatives all showed estrogenic activity. However, 2-hydroxydiphenyl and 3-hydroxydiphenyl showed lower activities than

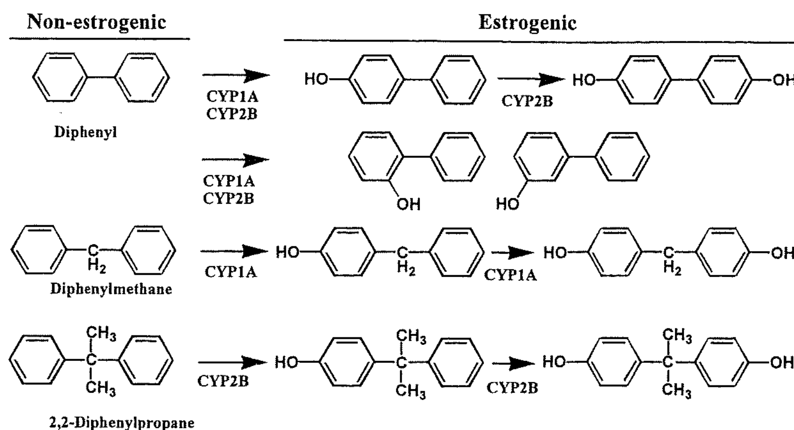

Fig. 4. Metabolic Pathways for the Activation of Diphenyl, Diphenylmethane and 2,2-Diphenylpropane to Estrogens by the Cytochrome P450 System

4-hydroxydiphenyl and 4,4′-dihydroxydiphenyl. Soto *et al.*[32] reported that 2-hydroxydiphenyl, which is used as an antifungal, is a weak estrogen, and the related compounds 4-hydroxydiphenyl and 3-hydroxydiphenyl are also estrogenic. Estrogenic activity of 4,4′-dihydroxydiphenyl has also been reported.[33] A possible metabolic activation pathway of these proestrogenic chemicals with liver microsomes is shown in Fig. 4. The estrogenic activity is likely to be mainly exhibited by the 4-hydroxyl derivatives, because the pathway leading from 4-hydroxyl derivatives to 4,4′-dihydroxyl derivatives does not proceed effectively in this metabolic system.

ACTIVATION OF STYRENE OLIGOMERS BY THE CYTOCHROME P450 SYSTEM

Styrene oligomers, such as *trans*-1,2-diphenylcyclobutane (TCB), *cis*-1,2-diphenylcyclobutane (CCB), 1,3-diphenylpropane, 2,4-diphenyl-1-butene, 2,4,6-triphenyl-1-hexene and 1α-phenyl-4β-(1′-phenylethyl)tetralin are incorporated into polystyrene resin as impurities in the course of manufacture, and may have a variety of biological actions, including hormonal activity.[34,35] Polystyrene has been used to manufacture food containers for take-out, such as coffee cups, meat trays, salad boxes and soup bowls, as well as instant food containers, in which instant foods such as Japanese noodles, buckwheat noodles, Chinese noodles, chow mein, spaghetti and rice are cooked by adding hot water. There are reports indicating that styrene oligomers migrate from these containers into the food contents.[36,37]

These styrene oligomers were negative in the estrogen screening assay. However, TCB exhibited estrogenic activity after incubation with liver microsomes of phenobarbital-treated rats in the presence of NADPH. CCB, 1,3-diphenylpropane and 2,4-diphenyl-1-butene also exhibited estrogenic activity after metabolic activation, but the activities were lower than that of TCB. 2,4,6-Triphenyl-1-hexene and 1α-phenyl-4β-(1′-phenylethyl)tetralin did not show estrogenic activity after such metabolic activation. After incubation of TCB with liver microsomal system of phenobarbital-treated rats, *trans*-1-(4-hydroxyphenyl)-2-phenylcyclobutane (4-OH-TCB), which exhibited a significant estrogenic activity, was detected. Recombinant human cytochrome P450 2B6 and rat cytochrome P450 2B1 were responsible for the activation. In contrast, cytochrome P450 1A may be mainly responsible for the activation of 2,4-diphenyl-1-butene. Thus, some styrene oligomers, especially TCB, exhibit estrogenic activity after metabolic activation to the 4-hydroxylated metabolite by rat liver microsomes (Fig. 5).[11]

Nobuhara *et al.* reported that styrene oligomers did not induce the proliferation of MCF-7 cells.[38] However, Ohyama *et al.* reported that some styrene dimers and trimers were estrogenic without metabolic activation in a cell proliferation assay with estrogen-responsive MCF-7 cells. They reported that TCB, CCB, 1,3-diphenylpropane and 2,4-diphenyl-1-butene were positive without

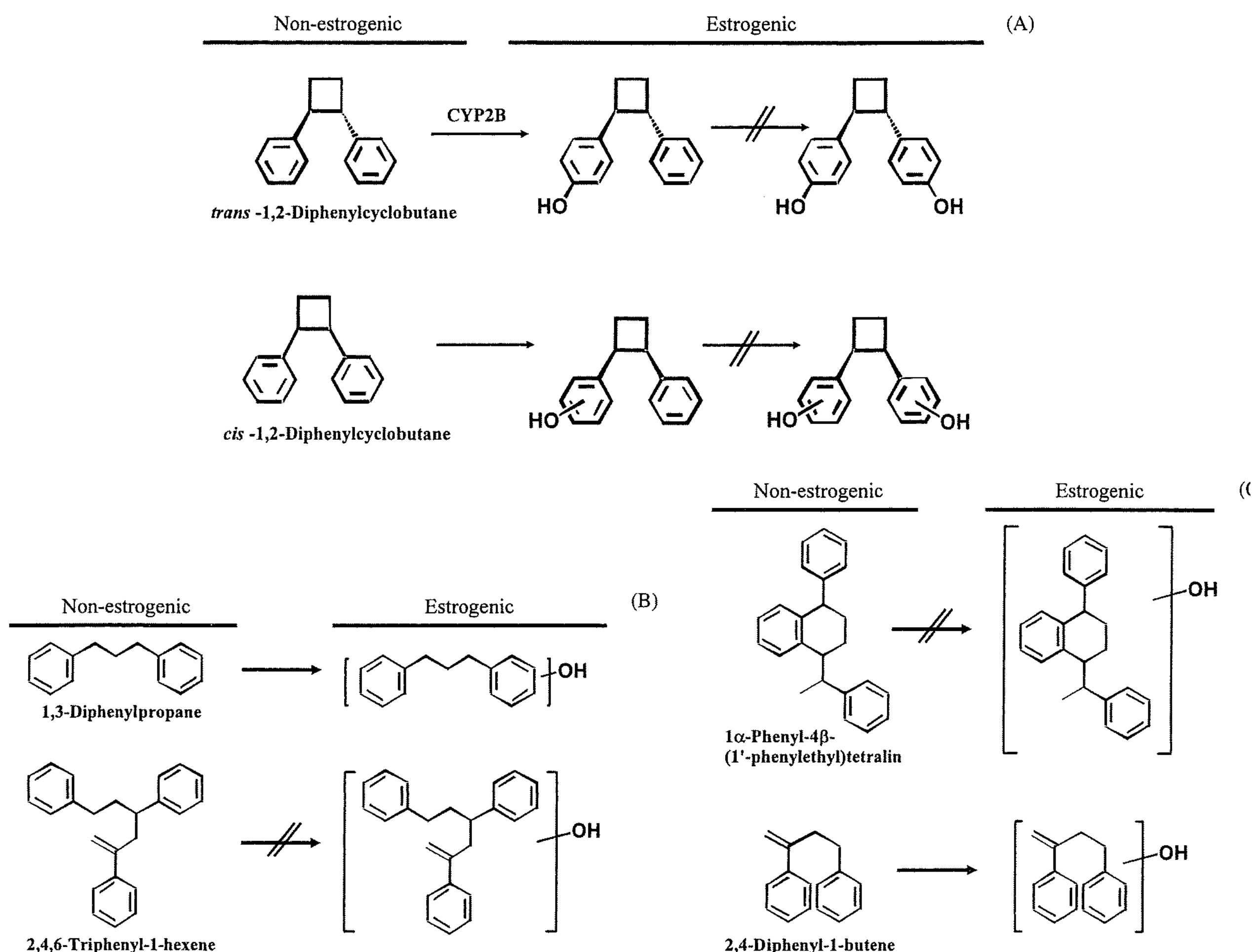

Fig. 5. Metabolic Pathways for the Activation of Styrene Oligomers to Estrogens by the Cytochrome P450 System

metabolic activation.[39] The difference from the above results was not clearly explained. However, one possibility is that, because the substrates were in contact with MCF-7 cells for a long time in their assay, estrogenic metabolites were generated in the cells.

A metabolic activation pathway of proestrogenic styrene oligomers with liver microsomes is shown in Fig. 5. TCB is converted to hydroxylated derivatives by rat liver microsomes. In the microsomal system, the estrogenic activity of TCB is thought to be mainly exhibited by 4-OH-TCB. The further oxidized metabolite, *trans*-1,2-bis-(4-hydroxyphenyl)cyclobutane, could not be detected. However, the activity of CCB after metabolic activation was lower than that of the *trans*-isomer. *cis*-1-(4-Hydroxyphenyl)-2-phenylcyclobutane may exhibit lower estrogenic activity than the *trans*-isomer. The activity of CCB after metabolic activation may be due to 4-OH-TCB formed after *cis*-*trans* isomerization.

ACTIVATION OF POLYCYCLIC AROMATIC HYDROCARBONS AND NITROPOLYCYCLIC AROMATIC HYDROCARBONS BY THE CYTOCHROME P450 SYSTEM

Some reports suggest that hydroxylated polycyclic aromatic hydrocarbons act as xenoestrogens.[40–42] Many polycyclic aromatic hydrocarbons present as environmental pollutants are negative in the estrogen screening test, but the possibility should be considered that these compounds may also be metabolically activated to xenobiotic estrogens via hydroxylation in the body. It was demonstrated that hydroxylated metabolites of benzo[*a*]pyrene exhibit estrogenic activity. In this case, the 1-, 3-, 7- and 9-hydroxylated metabolites had binding affinity for estrogen receptor, but the 7,8- and 9,10-dihydroxy metabolites did not.[40–42] De Wiele *et al.* also reported that polycyclic aro-

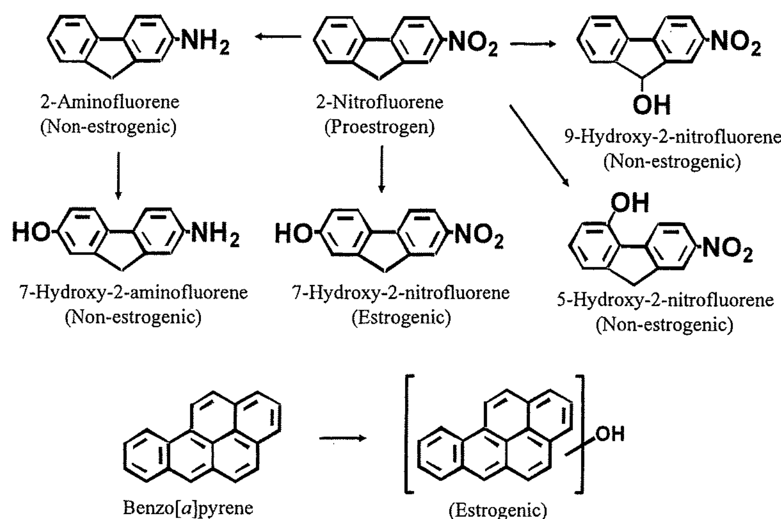

Fig. 6. Metabolic Pathways for the Activation of 2-Nitrofluorene and Benzo[*a*]pyrene to Estrogens by the Cytochrome P450 System

matic hydrocarbons were transformed to estrogenic hydroxylated metabolites by human colon microbiota (Fig. 6).[43]

Nitropolycyclic aromatic hydrocarbons (nitro-PAHs), which are carcinogenic and mutagenic, enter the environment from diesel engine exhaust, urban pollution sources, cigarette smoking and so on.[44–46] Several reports have indicated that nitro-PAHs are mainly metabolized by nitro reduction, ring hydroxylation, acylation and conjugation in mammalian species.[47] Nitro-PAHs should also be examined to see whether nitro reduction or hydroxylation of the aromatic rings activates these compounds to xenobiotic estrogens, as is the case for their carcinogenicity. 2-Nitrofluorene (NF) is a typical carcinogenic nitro-PAH.[48] NF was detected in diesel exhaust particles as a major component, together with nitropyrenes.[49] NF was also detected as a major pollutant in the atmosphere.[50] NF is a potent mutagen and forms DNA adducts in the animal body.[51–53] It was reported that NF is converted to 2-aminofluorene and its acylamino metabolites, and their oxidative metabolites.[54–57]

It was shown that NF exhibits a significant estrogenic activity after activation by rat liver microsomal mixed function oxidase. When the compound was incubated with the liver microsomes of 3-methylcholanthrene-treated rats in the presence of NADPH, 7-hydroxy-2-nitrofluorene (7-OH-NF) was formed as a major metabolite. However, little of the metabolite was formed by liver microsomes of untreated or phenobarbital-treated rats. Rat recombinant cytochrome P450 1A1/2 exhibited a significant oxidase activity toward NF, affording 7-OH-NF. 7-OH-NF exhibited a significant estrogenic activity, while the activity of 5-hydroxy-2-nitrofluorene was much lower.[12]

The estrogenic activity of NF was due to formation of the hydroxylated metabolite at the 7 position by liver microsomes. 2-Aminofluorene and 2-acetylaminofluorene did not exhibit estrogenic activity. These compounds had weak estrogenic activity after metabolic activation by liver microsomes of 3-methylcholanthrene-treated rats. NF is metabolized to hydroxylated derivatives, mainly 7-OH-NF, and is also converted to a reduced metabolite, 2-aminofluorene. In the microsomal system, the estrogenic activity of NF is thought to be mainly due to 7-OH-NF, because reductive metabolism of NF proceeds effectively only under anaerobic conditions. Further, it is possible that 7-OH-NF and 5-OH-NF are reduced to 7- and 5-hydroxy-2-aminofluorene, and acetylated to 7- and 5-hydroxy-2-acetylaminofluorene. However, these reactions are likely to be inactivation routes as regards estrogenic activity (Fig. 6).

ACTIVATION OF METHOXYCHLOR AND RELATED COMPOUNDS BY THE CYTOCHROME P450 SYSTEM

Methoxychlor is a proestrogen which requires demethylation by liver microsomal mixed function oxidase in animals prior to eliciting estrogenic ac-

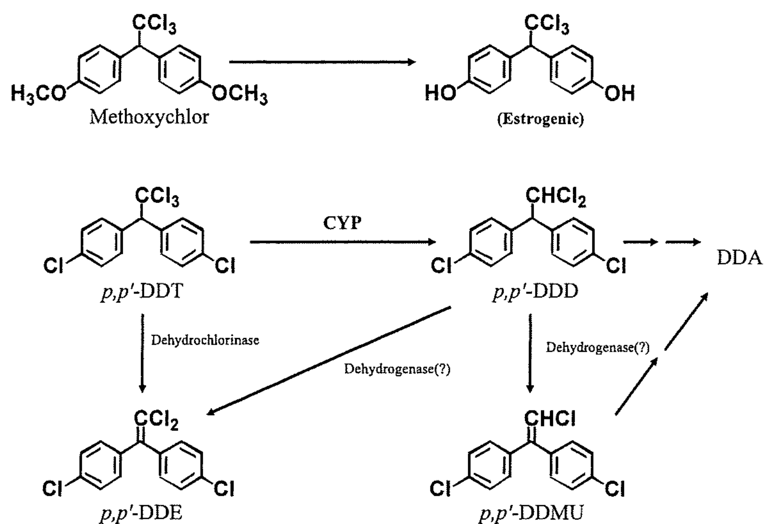

Fig. 7. Metabolic Pathways of Methoxychlor and *p,p′*-DDT in Rat Liver Microsomes

tivity.[13] Methoxychlor requires demethylation by liver microsomal mixed function oxidase, involving CYP 1A2 and 2C19, to elicit estrogenic activity.[58] Elsby *et al.* also reported that methoxychlor was activated through demethylation by human liver microsomes.[14] Schlenk *et al.* reported that methoxychlor was activated to estrogen in fish (Fig. 7).[59]

1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (*p,p′*-DDT) is metabolized to 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane (*p,p′*-DDD) and 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene (*p,p′*-DDE) by reductive dechlorination and dehydrochlorination, respectively, and *p,p′*-DDD is further oxidized to 2,2-bis(4-chlorophenyl)acetic acid (*p,p′*-DDA) in animals and fish (Fig. 7).[60–62] *p,p′*-DDD shows estrogenic activity and *p,p′*-DDE shows anti-androgenic activity.[63] Other DDT isomers exhibited similar endocrine-disrupting activity. The metabolites of *p,p′*-DDT and *o,p′*-DDT described above exhibited estrogenic activity (Fig. 7).[64–66] Nelson reported an *in vivo* uterotropic effect of some of these DDT metabolites.[67] Gray *et al.* reported that *p,p′*-DDT exhibited anti-androgenic activity, like *p,p′*-DDE.[68]

ACTIVATION OF α,β-UNSATULATED KETONES BY THE CYTOCHROME P450 SYSTEM

Naturally occurring phytoestrogens also show estrogenic activity *in vitro* in receptor binding assay, in spite of their beneficial effects, such as anticarcinogenicity.[69] Some flavonoids are phytoestrogens. Chalcones are a source of phytoestrogens, acting as C15 precursors in plant flavonoid biosynthesis.[70] *trans*-4-Phenyl-3-buten-2-one (PBO) also has a flavonoid skeleton. Chalcone (*trans*-1,3-diphenyl-2-propen-1-one) is an α,β-unsaturated ketone that has the skeleton of so-called "chalcones." They are also found in naturally occurring compounds, such as plant allelochemicals, insect hormones and pheromones.[71] PBO (also called *trans*-phenyl styryl ketone or benzalacetone) has a wide range of uses as a industrial material for synthesis of chemicals and drugs, and as a flavoring additive for cosmetics, soaps, detergents, cigarettes and foods.[72] α,β-Unsaturated ketones, in which the double bond is adjacent to the carbonyl group, are reactive compounds due to their electrophilic properties, and undergo nucleophilic attack, *e.g.*, with SH-groups in proteins. They exhibit genotoxicity and mutagenicity, as well as having anti-carcinogenic effects.[73–78]

Chalcone was converted to estrogenically active hydroxylated derivatives by rat liver microsomes. 4-Hydroxychalcone exhibited the highest activity. 4′-Hydroxychalcone and 2-hydroxychalcone were minor metabolites of chalcone, and 2′-hydroxychalcone was not formed. Their estrogenic activities were lower than that of 4-hydroxychalcone. The estrogenic activity of chalcone is thus thought to be mainly due to 4-hydroxychalcone, which is the major metabolite. PBO was also metabolically activated to an estrogen by a