The Estrogenicity of Bisphenol A-related Diphenylalkanes with Various Substituents at the Central Carbon and the Hydroxy Groups

Pilar Perez,[1] Rosa Pulgar,[2] Fátima Olea-Serrano,[2] Mercedes Villalobos,[1] Ana Rivas,[1] Manfred Metzler,[3] Vicente Pedraza,[1] and Nicolás Olea[1]

[1]Laboratory of Medical Investigation, Department of Radiology, School of Medicine, HUSC-University of Granada, Granada, Spain; [2]Department of Nutrition and Bromatology, School of Pharmacy, University of Granada, Granada, Spain; [3]Department of Food Chemistry, University of Karlsruhe, Karlsruhe, Germany

The chemical structure of hydroxylated diphenylalkanes or bisphenols consists of two phenolic rings joined together through a bridging carbon. This class of endocrine disruptors that mimic estrogens is widely used in industry, particularly in plastics. Bisphenol F, bisphenol A, fluorine-containing bisphenol A (bisphenol AF), and other diphenylalkanes were found to be estrogenic in a bioassay with MCF7 human breast cancer cells in culture (E-SCREEN assay). Bisphenols promoted cell proliferation and increased the synthesis and secretion of cell type-specific proteins. When ranked by proliferative potency, the longer the alkyl substituent at the bridging carbon, the lower the concentration needed for maximal cell yield; the most active compound contained two propyl chains at the bridging carbon. Bisphenols with two hydroxyl groups in the *para* position and an angular configuration are suitable for appropriate hydrogen bonding to the acceptor site of the estrogen receptor. Our data suggest that estrogenicity is influenced not only by the length of the substituents at the bridging carbon but also by their nature. Because diphenylalkane derivatives are widespread and their production and use are increasing, potential exposure of humans to estrogenic bisphenols is becoming a significant issue. The hazardous effects of inadvertent exposure to bisphenol-releasing chemicals in professional workers and the general populations therefore deserve investigation. *Key words*: bisphenol A, bisphenol F, endocrine-disrupting chemicals, hydroxylated diphenylalkanes, MCF7 breast cancer cells, xenoestrogens. *Environ Health Perspect* 106:167–174 (1998). [Online 5 February 1998]
http://ehpnet1.niehs.nih.gov/docs/1998/106p167-174perez/abstract.html

There is increasing evidence that organic molecules of different chemical structures, with a variety of industrial applications, are acting on living organisms and disrupting their endocrine systems (*1*). The chemical structure of one class of endocrine disruptors that mimic estrogens consists of two phenolic rings joined together through a bridging carbon. These diphenylalkanes have received the generic name bisphenols: bisphenol F (BPF) when the bridging carbon has no substituent, bisphenol A (BPA) when the bridging group contains two methyl groups, or bisphenol AF when the methyl groups of bisphenol A are perfluorinated (*2*).

Diphenylalkanes are currently among the leading chemicals in plastics (*3*), and BPA is a primary raw material for the production of polycarbonates, epoxy resins, phenolics resins, polyesters, and polyacrylates. Polycarbonates, which have become the most widely used material in engineered plastic, account for about 60% of total demand for BPA. In 1995, nearly 1.4 billion pounds ($\approx 700 \times 10^6$ kg) of this bisphenol were produced.

Epoxy resins, obtained by the reaction of bisphenols with epichlorohydrin, are the fundamental components of high commercial-quality polymer materials (*4*). These very transparent resins display good mechanical resistance and conserve their form at a wide range of temperatures, permitting the production of sterilizable materials. Epoxy-based coatings are used in many applications including product finishing, marine finishing, decorative floor manufacture, breaking petroleum emulsions, structural steel coating, lacquer coatings in cans and other vessels used for foodstuffs, tank coating, can and drum linings, and floor varnishes. They are also employed in dental composites and sealants and used as additives for a variety of other plastic materials such as vinyl and acrylic resins and natural and synthetic rubber.

In 1909, L.H. Baekeland patented the first phenolic plastic, known as bakelite (*3*). This material was produced by a reaction between phenol and formaldehyde which gives rise to BPF. Phenolic resins are produced by the copolymerization of simple phenols or bisphenols and formaldehyde. For example, polyformal II is made from perfluorinated BPA and formaldehyde (*5*). Coatings based on phenolic resins are tough, have excellent adhesion, and are used in inks, coatings, varnishes, and abrasive binders.

The first published reports on estrogenic effects of hydroxylated diphenylalkanes appeared in the 1930s. In 1936, Dodds and Lawson (*6*) reported the estrogenicity of some diphenyl compounds containing two hydroxyl groups in *para* positions. Reid and Wilson (*7*) subsequently confirmed the estrogenicity of 4,4′-dihydroxydiphenylalkane derivatives. In 1987, Morrisey et al. (*8*) analyzed the developmental toxicity of BPA in rats and mice. Citing a study conducted by Hardin from the National Institute for Occupational Safety and Health, Morrisey suggested that BPA exerted an estrogenic effect in ovariectomized rats. More recently, BPA released from polycarbonate flasks during autoclaving was also shown to have an estrogenic effect (*9*).

In recent years we identified routes of human exposure to BPA. We first demonstrated the presence of estrogenic activity in canned foodstuffs and identified the estrogenic component BPA as a chemical leaching from the inner plastic coating (*10*). BPA was present at concentrations ranging from 4 to 23 µg/can in both extracted foods and water from autoclaved cans. Subsequently, we demonstrated that the estrogenicity of some commercial composites and sealants used in dentistry was due to their BPA content. We found BPA and related compounds in saliva after standard dental treatments (*11*). Recently, we proposed to determine whether epoxy resins and polycarbonates contribute to the inadvertent and unsuspected exposure of humans to estrogenic xenobiotics (*12*).

As a follow-up of our previous work (*10,11*), we studied whether BPA-derived compounds with substitution of the hydroxy groups and the central carbon

Address correspondence to N. Olea, Department of Radiology, University of Granada, 18071-Granada, Spain.

The authors are grateful to Ana M. Soto and Carlos Sonnenschein of Tufts University for fruitful discussion and their critical review of the manuscript. The assistance of José Molina of the Organic Chemistry Department, University of Granada, is acknowledged. We thank Karen Shashok for editorial assistance. This research was supported by grants from the Spanish Ministry of Health (FIS, 95/1959) and the Andalusian Regional Government Department of Health (Consejería de Salud, 96/159).

This work was reported in part at the meeting Estrogens in the Environment IV: Linking Fundamental Knowledge, Risk Assessment, and Public Policy held in Washington, DC, 19–21 July 1997.

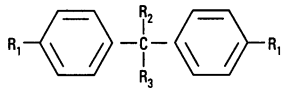
Received 31 July 1997; accepted 3 November 1997.

atom are estrogenic and tested the proliferative effect of BPA and structurally similar molecules commonly employed in plastic materials in MCF7 breast cancer cells. We also studied the potency of these compounds in inducing cell type-specific proteins (progesterone receptor and pS2) and their affinity to bind to the estrogen receptor extracted from immature rats.

Materials and Methods

Chemicals. 17β-estradiol (E_2) and diethylstilbestrol (DES) were purchased from Sigma Chemical Co. (St. Louis, MO). Various diphenylalkanes with substituents at the central carbon or the hydroxy groups were either purchased or synthesized by one of us (M.M.) (see Table 1 for structure). BPA, BPF, bisphenol A dimethacrylate (BisDMA), bisphenol A bischloroformate (BPACF), bisphenol A ethoxylate (E-BPA), bisphenol A propoxylate (P-BPA), bisphenol A ethoxylate diacrylate (BPA-EDA), bisphenol A diglycidylether (BADGE), and bisphenol A diglycidylether dimethacrylate (Bis-GMA) were obtained from Aldrich (Aldrich-Chemie, Albuch, Germany). 1,1-Bis(4-hydroxyphenyl)ethane (MM1); 1,1-bis(4-hydroxyphenyl)propane (MM2); 2,2-bis(4-hydroxyphenyl)butane (MM3); 3,3-bis(4-hydroxyphenyl)pentane (MM4); 4,4-bis(4-hydroxyphenyl)heptane (MM5); 2,2-bis(4-hydroxy-3-methylphenyl)propane (MM6); 2,2-bis(4- hydroxyphenyl)perfluoropropane (MM7 or bisphenol AF); bis(4-hydroxyphenyl)ketone (MM8); and 2,2-bis(4-hydroxyphenyl)propanol (MM9) were synthesized by M. Metzler. Purity of these chemicals was at least 97%. None of these contained bisphenol A according to HPLC and GC/MS analysis. Chemicals were dissolved in ethanol to a final concentration of 1 mM and stored at -20%C. [2,3,5-^{3}H]-E_2 (103 Bq/mmol) was obtained from Amersham (Buckinghamshire, England).

Table 1. Chemical characterization, proliferative effect, and induction of cell type-specific proteins in MCF7 cells, relative binding affinity to the estrogen receptor from rat uterine cytosol, and stoichiometric measurements of diphenylalkanes



Code	R_1	R_2	R_3	PE[a]	RPP[b]	RIPP[b]	RBA[c]	O-O[d]
E_2	NA	NA	NA	6.7 ± 1.2	100	100	100	10.96
BPF	-OH	-H	-H	7.1 ± 0.3	0.001	0.01	0.15	9.29
BPA	-OH	$-CH_3$	$-CH_3$	6.0 ± 0.5	0.01	0.1	0.056	9.20
BisDMA	$-OOC-C(CH_3)=CH_2$	$-CH_3$	$-CH_3$	5.6 ± 0.6	0.001	0.01	0.0015	8.87
BPACF	-OOCCl	$-CH_3$	$-CH_3$	4.6 ± 0.2	0.001	0.01	0.023	9.00
E-BPA	$-OCH_2-CH_2OH$	$-CH_3$	$-CH_3$	0.9 ± 0.1	na	na	na	9.30
P-BPA	$-OCH_2-CHOH-CH_3$	$-CH_3$	$-CH_3$	1.0 ± 0.3	na	na	na	9.28
BPA-EDA	$-OCH_2-CH_2-COO-CH=CH_2$	$-CH_3$	$-CH_3$	3.9 ± 0.6	0.0001	<0.01	0.0005	9.81
BADGE	$-OCH_2-CH(O)-CH_2$	$-CH_3$	$-CH_3$	2.0 ± 0.3	0.0001	na	na	9.31
BisGMA	$-OCH_2-CHOH-CH_2OOC-C(CH_3)=CH_2$	$-CH_3$	$-CH_3$	1.1 ± 0.1	na	na	na	8.80
MM1	-OH	$-CH_3$	-H	6.3 ± 0.4	0.001	0.01	0.0009	9.30
MM2	-OH	$-C_2H_5$	-H	6.7 ± 0.9	0.01	0.01	0.15	9.27
MM3	-OH	$-C_2H_5$	$-CH_3$	5.9 ± 0.2	0.01	0.1	0.15	9.27
MM4	-OH	$-C_2H_5$	$-C_2H_5$	7.2 ± 0.7	0.1	0.1	0.18	9.13
MM5	-OH	$-C_3H_7$	$-C_3H_7$	7.2 ± 0.8	1	1	0.15	9.12
MM6[e]	-OH	$-CH_3$	$-CH_3$	6.8 ± 0.6	0.01	0.1	0.25	9.65
MM7	-OH	$-CF_3$	$-CF_3$	5.5 ± 0.6	0.01	0.1	1	9.25
MM8	-OH	=O	-	5.5 ± 0.4	0.0001	<0.01	0.013	10.21
MM9	-OH	$-CH_2OH$	$-CH_3$	7.1 ± 0.4	0.0001	<0.01	0.0075	9.25

Abbreviations: PE, proliferative effect; RPP, relative proliferative potency; RIPP, relative induced protein potency; RBA, relative binding affinity; E_2, 17β-estradiol; BPF, bisphenol F; BPA, bisphenol A; BisDMA, bisphenol A dimethacrylate; BPACF, bisphenol A bischloroformate; E-BPA, bisphenol A ethoxylate; P-BPA, bisphenol A propoxylate; BPA-EDA, bisphenol A ethoxylate diacrylate; BADGE, bisphenol A diglycidyl ether; Bis-GMA, bisphenol A diglycidylether dimethacrylate; MM1, 1,1-bis(4-hydroxyphenyl)ethane; MM2, 1,1-bis(4-hydroxyphenyl)propane; MM3, 2,2-bis(4-hydroxyphenyl)butane; MM4, 3,3-bis(4-hydroxyphenyl)pentane; MM5, 4,4-bis(4-hydroxyphenyl)heptane; MM6, 2,2-bis(4-hydroxy-3-methylphenyl)propane; MM7, 2,2-bis(4-hydroxyphenyl)perfluoropropane; MM8, bis(4-dihydroxyphenyl)ketone; MM9, 2,2-bis(4-hydroxyphenyl)propanol; NA, not applicable; na, no activity.

[a]PE is expressed as the ratio between the highest cell yield obtained with the diphenylalkane tested and the hormone-free control.

[b]RPP and RIPP are 100 × the ratios between the dose of E_2 and that of the diphenylalkane needed to produce maximal cell yield and maximal expression of cell-type specific proteins, respectively. RPP and RIPP were set at 100 for E_2, which corresponded to 10 pM and 1 nM for maximal effect on cell yield and induction of cell-type specific proteins, respectively.

[c]RBA of each chemical was calculated as the ratio of the concentration of unlabeled E_2/competitor required to inhibit 50% of the specific [^{3}H]-E_2 binding, with the affinity of E_2 set at 100%.

[d]O-O is the measurement in Å of the distance between the two oxygen atoms adjacent to the ring.

[e]Two additional CH_3 groups in *ortho* positions to the hydroxy groups.

MCF7 cell line. The MCF7 breast cancer cell line was a gift from C. Sonnenschein (Tufts University, Boston, MA). For routine maintenance, cells were grown in Dulbecco's modification of Eagle's medium (DME) supplemented with 5% fetal bovine serum (FBS; PAA Labor und Forschungs Ges, MBH, Linz, Austria) in an atmosphere of 5% CO_2/95% air under saturating humidity at 37°C. The cells were subcultivated at weekly intervals using a mixture of 0.05% trypsin and 0.01 EDTA.

Charcoal–dextran treatment of serum. Plasma-derived human serum was prepared from outdated plasma by adding calcium chloride to a final concentration of 30 mM to facilitate clot formation. Sex steroids were removed from serum by charcoal–dextran stripping (*13*). Briefly, a suspension of 5% charcoal (Norit A, Sigma Chemical Co) with 0.5% dextran T-70 (Pharmacia-LKB, Uppsala, Sweden) was prepared. Aliquots of the charcoal–dextran suspension of a volume similar to the serum aliquot to be processed were centrifuged at 1,000 × *g* for 10 min. Supernatants were aspirated and serum aliquots were mixed with the charcoal pellets. This charcoal–serum mixture was maintained in suspension by rolling at 6 cycles/min at 37°C for 1 hr. The suspension was centrifuged at 1,000 × *g* for 20 min, and the supernatant was then filtered through a 0.20 µm filter (Gelman Sciences, Ann Arbor, MI). Charcoal dextran-treated human serum (CDHuS) was stored at -20°C until needed.

Cell proliferation experiments. MCF7 cells were used in the E-SCREEN test of estrogenicity according to a technique slightly modified (*14*) from that originally described by Soto et al. (*15*). Briefly, cells were trypsinized and plated in 24-well plates (Limbro, McLean, VA) at initial concentrations of 10,000 cells/well in 5% FBS in DME. Cells were allowed to attach for 24 hr; the seeding medium was then replaced with 10% CDHuS-supplemented phenol red-free DME. Different concentrations of the test compound were added, and the assay was stopped after 144 hr by removing medium from wells, fixing the cells, and staining them with sulforhodamine-B (SRB). The staining technique was modified from that described by Skehan et al. (*16*). Briefly, cells were treated with cold 10% trichloracetic acid and incubated at 4°C for 30 min, washed five times with tap water, and left to dry. Trichloroacetic-fixed cells were stained for 10 min with 0.4% (wt/vol) SRB dissolved in 1% acetic acid. Wells were rinsed with 1% acetic acid and air dried. Bound dye was solubilized with 10 mM Tris base (pH 10.5) in a shaker for 20 min.

Finally, aliquots were transferred to a 96-well plate and read in a Titertek Multiscan apparatus (Flow, Irvine, CA) at 492 nm. Linearity of the SRB assay with cell number was verified prior to cell growth experiments. Mean cell numbers from each experiment were normalized to the steroid-free control cultures to correct for differences in the initial seeding density. The proliferative effect (PE) was expressed as the ratio between the highest cell yield obtained with the diphenylalkane tested and the hormone-free control. The relative proliferative potency (RPP) was calculated as 100 × the ratio between the dose of E_2 and that of the diphenylalkane needed to produce maximal cell yield.

Progesterone receptor induction. MCF7 cells seeded in T25 flasks (Nunc, Roskilde, Denmark) were incubated in 10% CDHuS for 72 hr with 0.1–10 nM E_2, and a parallel set of flasks were exposed to 0.1–10 µM of the chemicals. Controls received the vehicle alone. At the end of the experiment, the medium was aspirated and the cells were frozen in liquid N_2. To extract receptor molecules, cells were incubated with 1 ml extraction buffer (0.5 M KCl, 10 mM potassium phosphate, 1.5 mM EDTA, and 1 mM monothioglycerol, pH 7.4) at 4°C for 30 min. Progesterone receptors (PgR) were measured in extracted cells by enzyme immunoassay using the Abbott progesterone receptor kit (Abbott Diagnostics, Chicago, IL) according to the manufacturer's instructions.

Induction and secretion of pS2. pS2 was measured in the culture medium of MCF7 cells with the ELSA-pS2 immunoradiometric assay (CIS Bio International, Gif-sur-Yvette, France). Cells were subcultured into 24-well plates for 144 hr in 10% CDHuS. The culture medium was centrifuged at 1,200 × *g* for 10 min to eliminate floating and detached cells. Results are expressed as nanograms of secreted protein per million cells. The relative induced protein potency (RIPP) was calculated as 100 × the ratio between the dose of E_2 and that of the diphenylalkane needed to produce maximal expression of cell-type specific proteins (pS2 and PgR).

Competitive binding assay. Cytosol from immature female rat uteri was prepared at a protein concentration of approximately 2 mg/ml in phosphate buffer. Aliquots of this supernatant were then incubated with various concentrations of chemicals and 3 nM [^{3}H]-estradiol for 16 hr at 0–4°C. The free and bound fractions were separated with the charcoal–dextran technique. The relative binding affinity (RBA) of each competitor was calculated as the ratio of the concentration of unlabeled estradiol/competitor required to inhibit 50% of the specific [^{3}H]-E_2 binding, with the affinity of estradiol set at 100%.

Structural analysis. The Hyperchem software package (Hyperchem v. 3 for Windows, 1993; Autodesk Inc, Sausalito, CA) was used for molecular modeling studies. Natural (E_2) and synthetic estrogens (DES) as well as diphenylalkanes were constructed and then geometrically optimized to achieve the minimal energy conformation using MM+ force fields and to measure the distance between the oxygen groups.

Statistical analysis. Results were expressed as the mean ± standard deviation (SD). Proliferation yield experiments conducted in quadruplicate wells were repeated at least three times. Mean cell numbers from

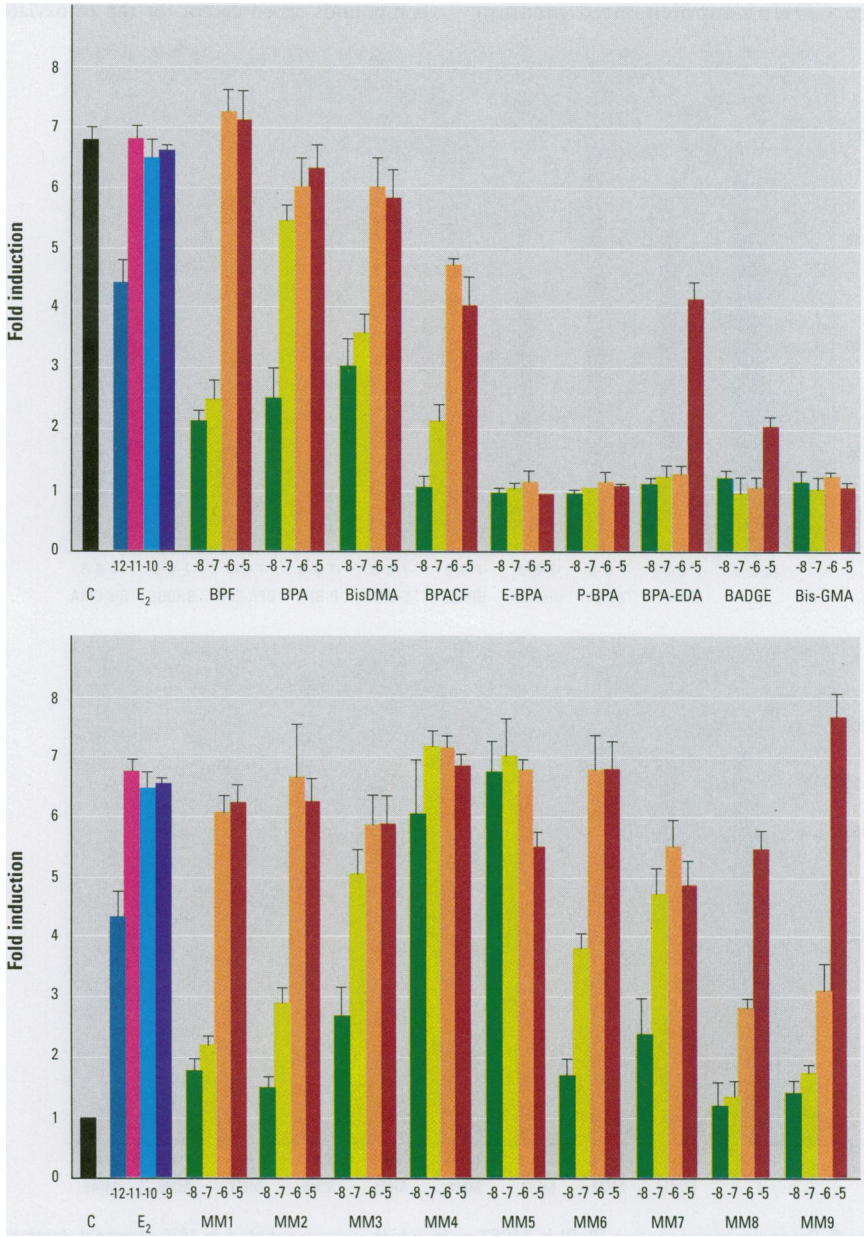


Figure 1. Proliferation (quadruplicate values and standard deviations) of MCF7 cells grown in 10% charcoal dextran-treated human serum-supplemented medium exposed for 144 hr to E_2 and to diphenylalkanes. Abbreviations: C, control; E_2, 17β-estradiol; BPF, bisphenol F; BPA, bisphenol A; BisDMA, bisphenol A dimethacrylate; BPACF, bisphenol A bischloroformate; E-BPA, bisphenol A ethoxylate; P-BPA, bisphenol A propoxylate; BPA-EDA, bisphenol A ethoxylate diacrylate; BADGE, bisphenol A diglycidyl ether; Bis-GMA, bisphenol A diglycidylether dimethacrylate; MM1, 1,1-bis(4-hydroxyphenyl)ethane; MM2, 1,1-bis(4-hydroxyphenyl)propane; MM3, 2,2-bis(4-hydroxyphenyl)butane; MM4, 3,3-bis(4-hydroxyphenyl)pentane; MM5, 4,4-bis(4-hydroxyphenyl)heptane; MM6, 2,2-bis(4-hydroxy-3-methylphenyl)propane; MM7, 2,2-bis(4-hydroxyphenyl)perfluoropropane; MM8, bis(4-dihydroxyphenyl)ketone; MM9, 2,2-bis(4-hydroxyphenyl)propanol. The x-axis represents concentrations 10^{-12} (-12), 10^{-11} (-11), etc. Controls received vehicle alone; E_2 was effective at concentrations as low as 1 pM (0.272 pg/ml).

each experiment were normalized to the steroid-free control to correct for differences in the initial seeding density. Differences between the different chemical treatment groups were assessed by analysis of variance and the *a posteriori* Shaffe's test. A *p*-value of ≤0.05 was regarded as significant.

Results

Cell proliferation experiments. Addition of E_2 to CDHuS-supplemented medium increased the number of MCF7 cells in the culture. Maximum proliferative effect was obtained at concentrations of 10 pM E_2 and higher (Fig. 1). Cell yields were sixfold greater than in control cultures after 6 days (mean ± SD = 6.67 ± 1.21 in 15 experiments). In the absence of E_2 (control), cells proliferated minimally. The PE of diphenylalkanes is shown in Figure 1 and Table 1. PE was significantly different from 1 for all the compounds tested except for the ethoxylate and propoxylate of BPA (E-BPA and P-BPA) and Bis-GMA, which were negative in the bioassay. In comparison with the PE of E_2, all these compounds showed a full agonistic response except BPACF, BPA-EDA, and BADGE, which produced cell yields below 60% of those found with E_2. Therefore, they behaved as partial estrogen agonists at the maximal concentration assayed (10 µM). In addition, estimated relative proliferative potency (RPP) allowed us to rank diphenylalkanes by their estrogenic potency: E_2>MM5>MM4>BPA, MM2, MM3, MM6, MM7>BPF, MM1, BPACF, Bis-DMA>MM8, MM9, BPA-EDA, BADGE.

Induction of progesterone receptor. The basal concentration of estrogen receptors (ER) and PgR in MCF7 cells was 183 ± 29 fmol/mg and 15.5 ± 4.3 fmol/mg of extracted protein, respectively. E_2 increased the concentration of PgR nearly 10–15-fold over the basal value (Fig. 2). Exposure of MCF7 cells to diphenylalkanes and related compounds resulted in a significant increase in PgR (Fig. 2). With the exception of MM8, MM9, E-BPA, P-BPA, and Bis-GMA, all other chemicals tested increased the levels of the PgR to a degree similar to that found with the natural estrogen. However, the concentration needed for maximal effect ranged from 0.1 µM for MM5, the most active compound, to 10 µM for the others. A good statistical correlation was found between the ability of these chemicals to induce PgR and to increase MCF7 cell yields in the proliferation bioassay (r = 0.908). With the exception of BADGE, which showed the poorest proliferative effect and was unable to induce PgR at the maximal concentration tested (10 µM), all other chemicals that induced PgR were also effective in the proliferation bioassay. In addition, the induction of PgR for MM8 and MM9 was not maximal although they were full agonists for proliferation.

pS2 Secretion. pS2 Secretion by MCF7 cells was significantly increased by concentrations ≥0.1 nM E_2 (≈3.5-fold increase over controls). The basal concentration of pS2 (53.6 ± 8.7 ng/10^6 cells) increased to 179.4 ± 13.1 ng/10^6 cells after treatment with 1 nM E_2 (Fig. 3). Exposure of MCF7 cells for 144 hr to various diphenylalkanes resulted in a significant increase in secreted pS2 (Fig. 3). A good statistical correlation was found between the ability of these chemicals to induce pS2 and to increase MCF7 cell yields in the proliferation bioassay (r = 0.809). Both E-BPA and P-BPA, as well as bis-GMA and BADGE, were ineffective in the induction and secretion of pS2. BPA-EDA was fully inductory for pS2 and partially for PgR and cell proliferation.

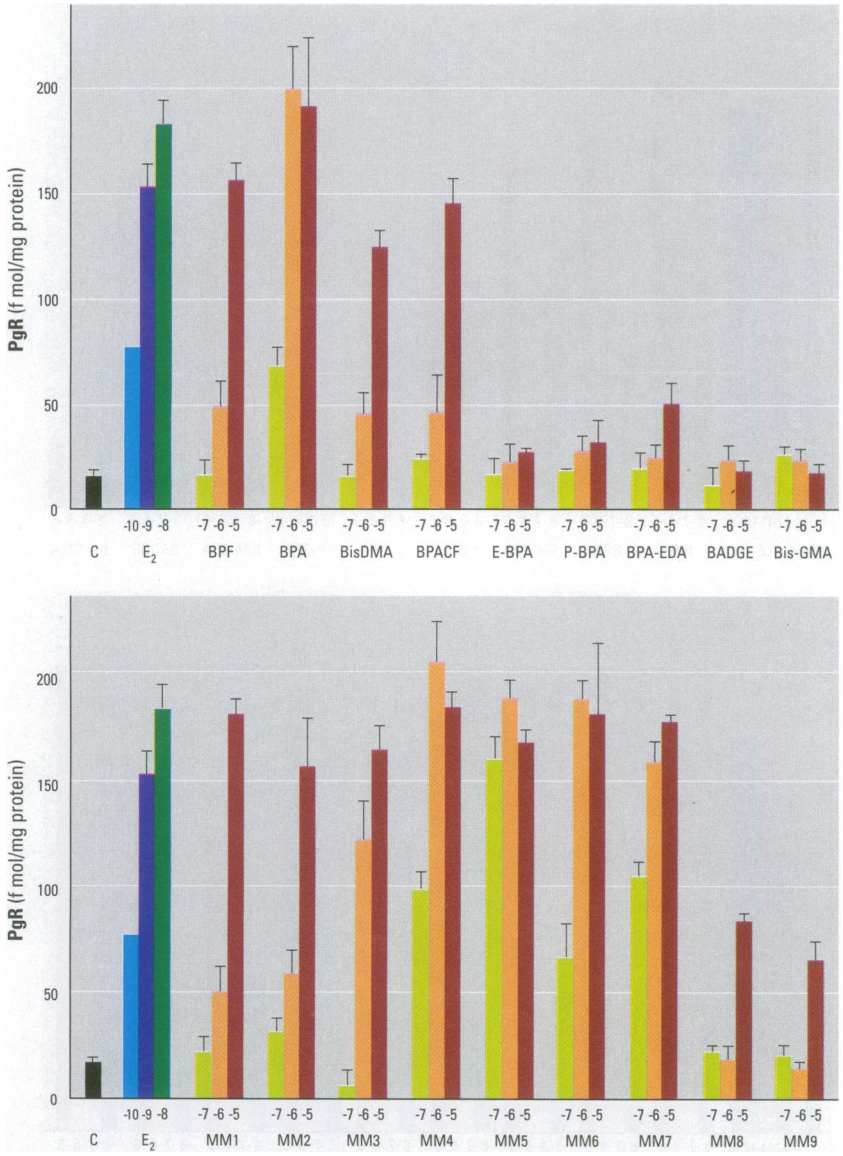


Figure 2. Progesterone receptors (PgR) in MCF7 cells, which were incubated in 10% charcoal dextran-treated human serum for 72 hr with 10^{-10} (-10), 10^{-9} (-9), and 10^{-8} (-8) M E_2. A parallel set of flasks were exposed to 10^{-7} (-7), 10^{-6} (-6), and 10^{-5} (-5) M diphenylalkanes. Abbreviations: C, control; E_2, 17β-estradiol; BPF, bisphenol F; BPA, bisphenol A; BisDMA, bisphenol A dimethacrylate; BPACF, bisphenol A bischloroformate; E-BPA, bisphenol A ethoxylate; P-BPA, bisphenol A propoxylate; BPA-EDA, bisphenol A ethoxylate diacrylate; BADGE, bisphenol A diglycidyl ether; Bis-GMA, bisphenol A diglycidylether dimethacrylate; MM1, 1,1-bis(4-hydroxyphenyl)ethane; MM2, 1,1-bis(4-hydroxyphenyl)propane; MM3, 2,2-bis(4-hydroxyphenyl)butane; MM4, 3,3-bis(4-hydroxyphenyl)pentane; MM5, 4,4-bis(4-hydroxyphenyl)heptane; MM6, 2,2-bis(4-hydroxy-3-methylphenyl)propane; MM7, 2,2-bis(4-hydroxyphenyl)perfluoropropane; MM8, bis(4-dihydroxyphenyl)ketone; MM9, 2,2-bis(4-hydroxyphenyl)propanol. Controls received vehicle alone. At the end of the experiment, the medium was discarded and flasks were kept in liquid N_2 until assayed. PgR was measured in extracted cells with a monoclonal antibody technique as indicated in Methods. Results are expressed as femtomol per milligram of extracted protein ± standard deviation.

Relative binding affinities. Relative binding affinities (RBA) for ER of diphenylalkanes ranged from 1 (MM7) to 0.0005 (BPA-EDA) (Fig. 4; Table 1). As suspected, E-BPA, P-BPA, and Bis-GMA showed no affinity for binding to the ER, even at concentrations 1 million-fold higher than those of E_2. A good relationship was found between the RBA estimated in the uterine cytosol assay and the proliferative potencies of each compound using the MCF7 breast cancer cell proliferation bioassay (r = 0.842). This result suggested that the proliferative effect of diphenylalkanes was mediated through their binding to the ER. However, a discrepancy between receptor affinity and proliferative ability was observed; BADGE was a partial agonist in the E-SCREEN assay, showing no affinity for binding to the uterine ER.

Chemical structure. Structures of natural and synthetic estrogens and estrogenic xenobiotics can be compared by measuring the distance between the two oxygen atoms responsible for hydrogen bonding to the acceptor site of the estrogen receptor. The O(3)-O(17) interatomic distance for E_2 was 10.96 Å, and the O(4)-O(4) distance of the synthetic estrogen DES was 12.1 Å. The various diphenylalkanes listed in Table 1 showed an O(4)-O(4) interatomic distance that ranged from 8.80 to 10.21 Å, slightly less than in E_2. Differences in estrogenic activity among the diphenylalkyls tested seemed to be dependent on the type of radical incorporated to the bridging carbon and the distance between the bonding groups.

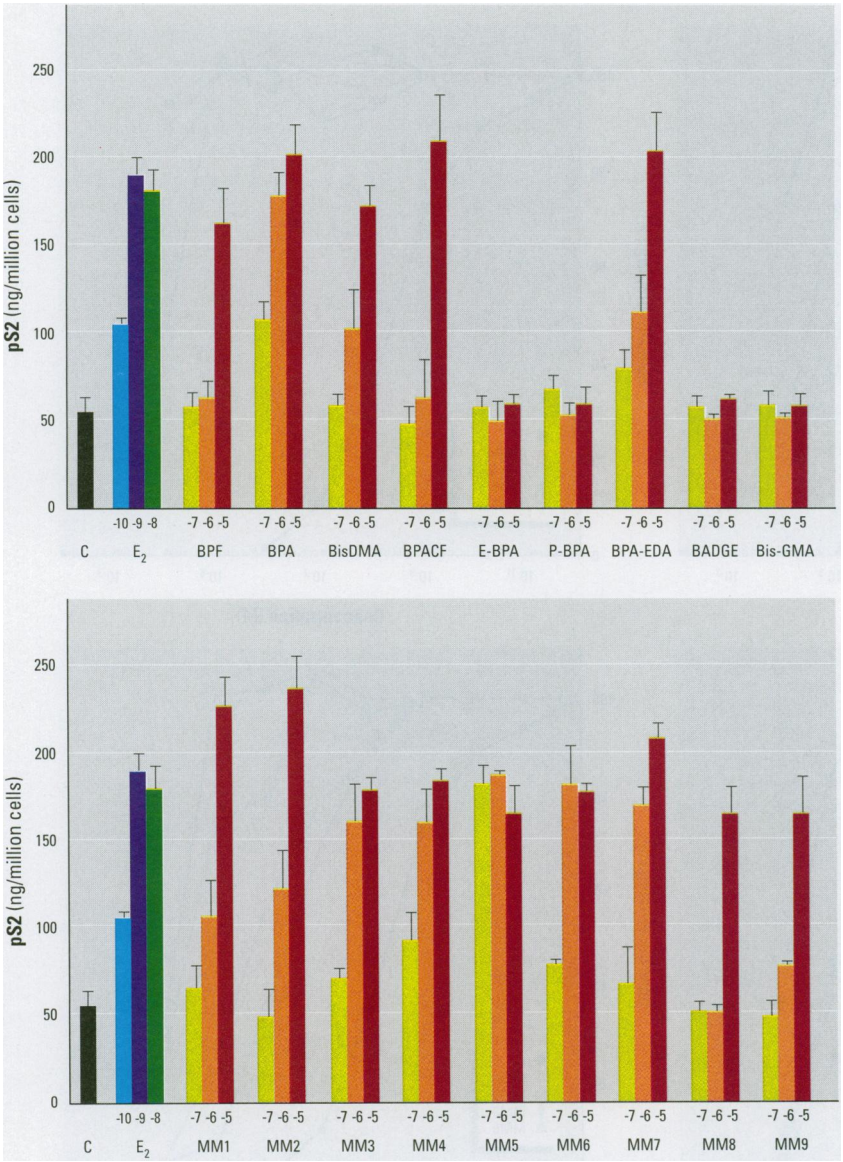


Figure 3. Accumulation of pS2 in culture medium. MCF7 cells were grown in 10% charcoal dextran-treated human serum-supplemented medium and exposed for 144 hr to 10^{-10} (-10), 10^{-9} (-9), and 10^{-8} (-8) M E_2. Parallel cultures were exposed to 10^{-7} (-7), 10^{-6} (-6), and 10^{-5} (-5) M diphenylalkanes. Abbreviations: C, control; E_2, 17β-estradiol; BPF, bisphenol F; BPA, bisphenol A; BisDMA, bisphenol A dimethacrylate; BPACF, bisphenol A bischloroformate; E-BPA, bisphenol A ethoxylate; P-BPA, bisphenol A propoxylate; BPA-EDA, bisphenol A ethoxylate diacrylate; BADGE, bisphenol A diglycidyl ether; Bis-GMA, bisphenol A diglycidylether dimethacrylate; MM1, 1,1-bis(4-hydroxyphenyl)ethane; MM2, 1,1-bis(4-hydroxyphenyl)propane; MM3, 2,2-bis(4-hydroxyphenyl)butane; MM4, 3,3-bis(4-hydroxyphenyl)pentane; MM5, 4,4-bis(4-hydroxyphenyl)heptane; MM6, 2,2-bis(4-hydroxy-3-methylphenyl)propane; MM7, 2,2-bis(4-hydroxyphenyl)perfluoropropane; MM8, bis(4-dihydroxyphenyl)ketone; MM9, 2,2-bis(4-hydroxyphenyl)propanol. Controls received vehicle alone. Results are expressed as nanograms per million cells ± standard deviation.

Discussion

BPF, BPA, bisphenol AF, and other diphenylalkanes are estrogenic in MCF7 cells, promoting cell proliferation and increasing the synthesis and secretion of cell-type specific proteins. When ranked by proliferative potency, the longer the alkyl substituent at the bridging carbon, the lower the concentration needed to give maximal cell yield; the most active molecule was that with two propyl chains at the central carbon, i.e., MM5. These results are in agreement with those reported in 1944 by Reid and Wilson (*7*), who showed the propylpropyl derivative, within a wide series of 4,4′-dihydroxydiphenylmethane derivatives, as the most potent estrogenic compound *in vivo*, when using the uterine test.

Dodds and Lawson (*6*) were the first to note that the phenanthrene condensed-ring structure was not necessary for estrogenic activity. Recently, by studying correspondence factor analysis to structure–activity relationships, Gilbert et al. (*17*) suggested that, in the diphenylalkanes, both phenyl rings are needed to display substantial proliferative activity in MCF7 cells and that the phenyl groups should be separated by at least a single carbon or preferably by two carbons. However, the estrogenicity of some alkylphenols bearing only one phenyl ring was demonstrated some years ago by Soto et al. (*18*), who showed that alkylphenols with at least a three-carbon alkyl chain in the *para* position had a proliferative effect on MCF7 cells (*19*). Soto and coworkers (*19*) also showed that fused rings such as naphthols are not estrogenic, despite being an integral part of the A and B ring of natural steroids and that some polychlorinated biphenyls promoted proliferation in MCF7 cells.

Among factors that make a molecule estrogenic, Leclercq (*20*) found that the existence of an oxygen functional group (hydroxylic, ketonic, or carbonyl) located at the end of the molecule opposite the phenolic ring conferred or at least amplified estrogenicity. It is now clear that this

group is present in the doisynolic acid derivatives as a carbonyl group (*19*) and in DDT and some polychlorinated biphenyls as chlorine residues susceptible to hydroxylation (*21*). In terms of chemical structure, the diphenylalkanes assayed in this study carry two terminal hydroxyl groups in the *para* position, either free (BPA, BPF, and MM1 to MM9) or as ether or ester bonds. Ester derivatives of diphenylakanes (Bis-DMA and BPACF) were estrogenic in the proliferation and protein induction assays, in contrast to some ether substituents at the terminal -OH (E-BPA, P-BPA, and Bis-GMA) that showed no estrogenic activity in the range of concentrations tested.

It has been pointed out that the proliferative activity of diphenylalkanes is reduced when the *para*-hydroxy groups are replaced by diethylaminoethoxy substituents (*17*), but experimental data suggest that ester and ether type bonds can be cleaved by MCF7 cells, releasing *para*-hydroxy groups and activating their estrogenicity. This seemed to be the case for BADGE and BPA-EDA, which were estrogenic in the proliferation bioassay at high concentrations (10 µM) even though they showed very low (BPA-EDA) and no binding affinity (BADGE) for the estrogen receptor. Thus estrogenicity could be related to the ability of cellular enzymatic systems to break down these bonds and to generate molecules with free hydroxyl groups. Metabolization of BADGE to release a more active compound in the E-SCREEN assay suggests that the results of receptor binding assays should be considered with caution when assessing estrogenicity. More recently Mariotti et al. (*22*) reported

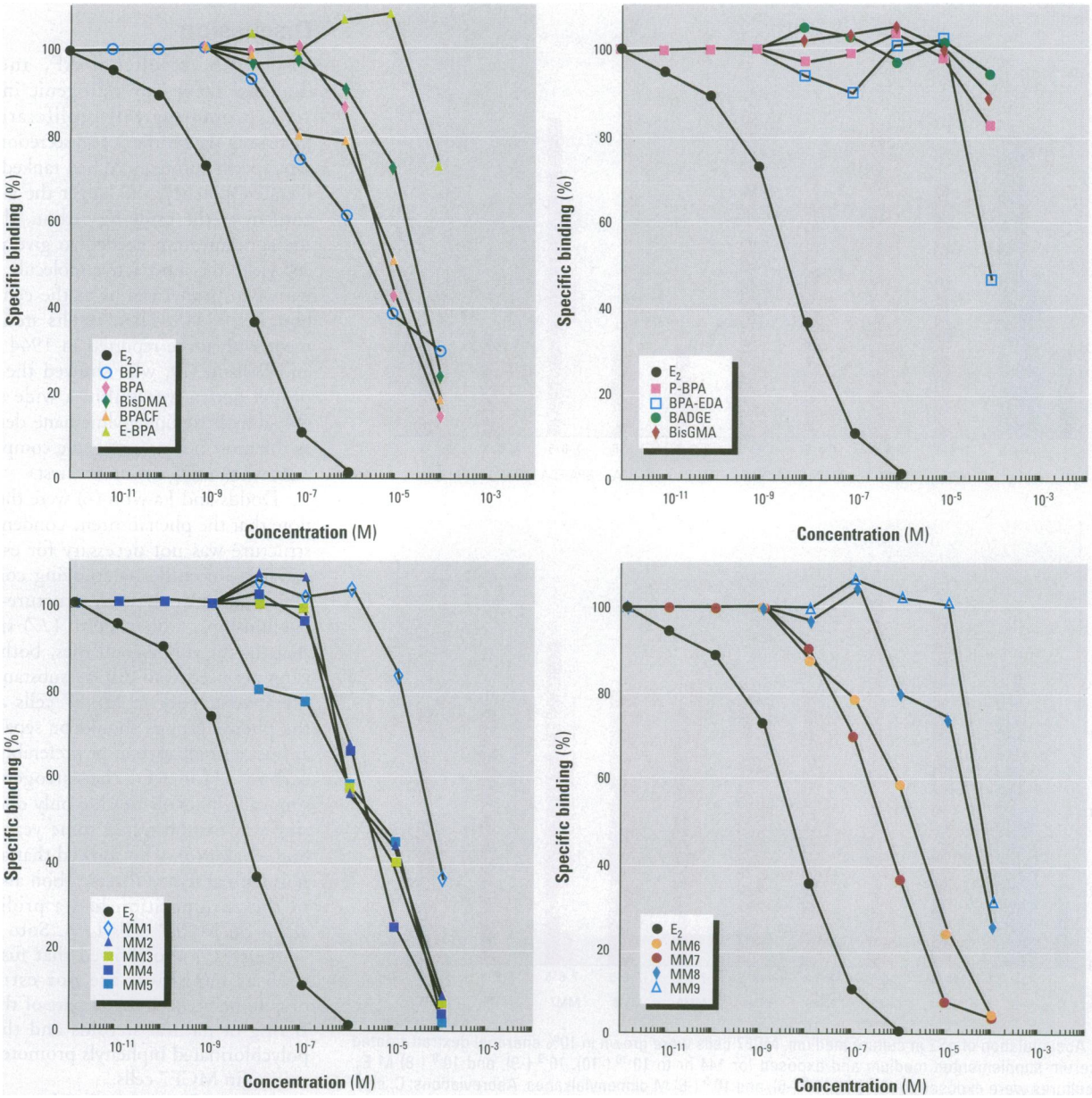


Figure 4. Competitive displacement of [^{3}H]-E_2 from the estrogen receptor by unlabeled E_2 and diphenylalkanes. Abbreviations: E_2, 17β-estradiol; BPF, bisphenol F; BPA, bisphenol A; BisDMA, bisphenol A dimethacrylate; BPACF, bisphenol A bischloroformate; E-BPA, bisphenol A ethoxylate; P-BPA, bisphenol A propoxylate; BPA-EDA, bisphenol A ethoxylate diacrylate; BADGE, bisphenol A diglycidyl ether; Bis-GMA, bisphenol A diglycidylether dimethacrylate; MM1, 1,1-bis(4-hydroxyphenyl)ethane; MM2, 1,1-bis(4-hydroxyphenyl)propane; MM3, 2,2-bis(4-hydroxyphenyl)butane; MM4, 3,3-bis(4-hydroxyphenyl)pentane; MM5, 4,4-bis(4-hydroxyphenyl)heptane; MM6, 2,2-bis(4-hydroxy-3-methylphenyl)propane; MM7, 2,2-bis(4-hydroxyphenyl)perfluoropropane; MM8, bis(4-dihydroxyphenyl)ketone; MM9, 2,2-bis(4-hydroxyphenyl)propanol. The estimated relative binding affinities of diphenylakanes are shown in Table 1. [^{3}H]-E_2 concentration was 3.8 nM, total binding was 58 fmol/mg protein, and the dissociation constant $K_d = 1.2 \times 10^{-10}$ M.

the estrogenicity *in vivo* of Bis-GMA using the uterotropic assay.

Lewis et al. (*23*) postulated that two hydroxy groups of E_2 take part in binding to the acceptor site of the estrogen receptor by hydrogen bonding. Various diphenylalkanes with two hydroxyl groups but different substituents at the central carbon atom that cause different angular configuration were tested in our study. In view of the structural similarities of the molecules assayed, we performed a computer-based study of the structure–function relationships. Although this could be a useful alternative, current knowledge does not allow the estrogenic effect of xenobiotics to be dependably predicted on the basis of structural data. Nevertheless, the combination of biological assays and structural analyses allows us to draw some preliminary conclusions (*24,25*).

Our experimental data suggested that not only the distance between *para*-hydroxy groups but also the nature of the bridging carbon substituents may determine estrogenicity. Among the MM compounds, for example, MM8 [bis(4-hydroxyphenyl)ketone] is more polar than the other compounds because of its carbonyl group; this compound showed the poorest proliferative effect, which implies that a lower polarity enhances estrogenicity. No single structural feature seems to define estrogenic activity; as proposed by Gilbert et al. (*17*), hydrophobic volume together with hydroxy groups and conjugation with basic groups are involved in the triggering of cell proliferation.

The introduction of methyl groups in the *meta* position of the aromatic ring, i.e., MM6, did not modify estrogenicity. This suggests that the effect of methyl substituents in bisphenols is not comparable to the dramatic reduction of estrogenicity caused by the introduction of methyl groups into 2- and 4- position of the A-ring of E_2 (*26*).

The estrogenicity of bisphenol polymers may be due to their nonpolymerized monomers or by chemical degradation. Polycarbonates and epoxy resins can be mechanically and thermally degraded, and these are major problems in their application (*27*). Scrap polycarbonate resins can be depolymerized by heating in an alkaline milieu to generate BPA (*28*). In fact, BPA was released from polycarbonate flasks (*9*) and resin-coated food cans and containers (*10,29*) during autoclaving and from incompletly cured resins (*11*). Our results suggest that BADGE, a monomer of epoxy resins, becomes estrogenic at a high concentration (10 μM), even before hydrolytic treatment. BADGE and BPF diglycidyl ether (BFDGE) have short half-lifes (less than 2 days) in acidic media, and half-life decreases further with increasing temperature (*30*). These findings suggest that the biological activity of by-products of BADGE and BFDGE should be considered when toxicity of the parent compounds is being assessed.

Little is known about the metabolism of BPA polymers or BPA itself in animals. More attention has been paid to epoxy compounds because of their alkylating properties (*31–34*). Interestingly, Climie et al. (*35,36*) studied the metabolic degradation of [^{14}C]-BADGE in mice after oral administration of a single dose. They found that 90% of the radioactivity was eliminated in the feces and urine during the first 3 days of the experiment. A small amount of BADGE (≈5%) underwent oxidative dealkylation to give glycidaldehyde (which has alkylating properties) and BPA, among other products. The systemic behavior of BPA and other bisphenols is poorly known and needs further investigation.

The environmental biodegradation of BPA by strain MV1 of Gram-negative bacteria was recently studied (*37,38*). This bacteria is able to grow in media with BPA as the only carbon source. The products of biodegradation include 60% CO_2 and 20% of phenolic derivatives such as 4-hydroxybenzoic acid, 4-hydroxybenzophenone, and molecules such as *trans*-4-4′dihydroxystilbene, clearly identified with high performance liquid chromatography. Although these processes have been tested only in microorganisms, they reflect a new relationship between BPA and molecules with putative estrogenic effect.

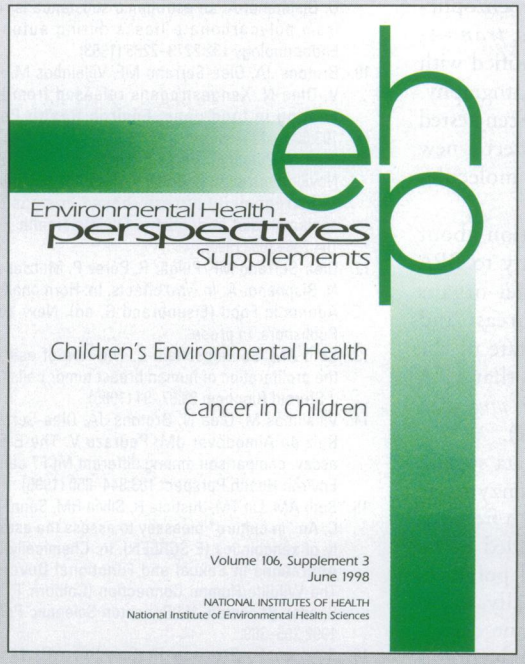
Recently, valuable information about genetic differences in suceptibility to BPA (*39*), effects on new BPA-target organs beyond the obvious ones of breast and uterus, and effects on the prostate of the developing fetus (*40*) indicate that BPA appears to be more estrogenic *in vivo* than predicted in *in vitro* assays (*41,42*).

In summary, experimental data suggest that thermal, chemical, and enzymatic degradation of diphenylalkanes is a frequent event. Unpolymerized and degraded BPF-, BPA-, and bisphenol AF-based polymers represent a source of biologically active monomers. Because diphenylalkane derivatives are widespread and their production is increasing, potential exposure to estrogenic diphenylalkanes both in the workplace and the home environment is becoming a significant issue. The hazardous effects of continuous exposure to bisphenol-releasing chemicals in exposed workers and in general populations demand investigation. We need to know to what extent human populations are exposed to bisphenols, how much is absorbed daily, how these compounds behave inside the human body, how they are metabolized, and what effects they have on human health. Meanwhile, diphenylalkanes should be regarded as estrogenic xenobiotics, and measures against inadvertent exposure should be implemented.

References

1. Colborn T, Vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101:378–384 (1993).
2. Dermer OC. Bisphenol A. In: Encyclopedia of Chemical Processing and Design (McKelta JJ, ed). New York:Marcel Dekker, 1983;406–430.
3. Smith WF. Fundamentos de la Ciencia e Ingeniería de Materiales. 2nd ed. New York:McGraw-Hill, 1994.
4. Bauer RS, De La Mare HE, Klarquist JM, Newman SF. Epoxy resins and epoxides. In: Encyclopedia of Chemical Processing and Design (McKelta JJ, ed). New York:Marcel Dekker, 1983;261–297.
5. Nakamura S, Suzuki Y, Kojima T, Tago K. Thermal degradation of aromatic polyformals derived from bisphenol A and bisphenol AF. Thermochimica Acta 267:231–237 (1995).
6. Dodds EC, Lawson W. Synthetic estrogenic agents without the phenanthrene nucleus. Nature 137:996 (1936).
7. Reid EE, Wilson E. The relation of estrogenic activity to structure in some 4,4′-dihydroxydephenylmethanes. J Am Chem Soc 66:967–968 (1944).
8. Morrissey RE, George JD, Price CJ, Tyl RW, Marr MC, Kimmel CA. The developmental toxicity of bisphenol-A in rats and mice. Fundam Appl Toxicol 8:571–582 (1987).
9. Krishnan AV, Stathis P, Permuth SF, Tokes L, Feldman D. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology 132:2279–2286 (1993).
10. Brotons JA, Olea-Serrano MF, Villalobos M, Pedraza V, Olea N. Xenoestrogens released from lacquer coating in food cans. Environ Health Perspect 103:608–612 (1995).
11. Olea N, Pulgar R, Pérez P, Olea-Serrano F, Rivas A, Novillo-Fertrell A, Pedraza V, Soto AM, Sonnenschein C. Estrogenicity of resin-based composites and sealants used in dentistry. Environ Health Perspect 104:298–305 (1996).
12. Olea-Serrano MF, Pulgar R, Pérez P, Metzler M, Olea N. Bisphenol-A: *In vitro* effects. In: Hormonally Active Agents in Food (Eisenbrand G, ed). New York:VCH Publishers. In press.
13. Soto AM, Sonnenschein C. The role of estrogen on the proliferation of human breast tumor cells (MCF-7). J Steroid Biochem 23:87–94 (1985).
14. Villalobos M, Olea N, Brotons JA, Olea-Serrano MF, Ruiz de Almodóvar JM, Pedraza V. The E-SCREEN assay: comparison among different MCF7 cell stocks. Environ Health Perspect 103:844–850 (1995).
15. Soto AM, Lin TM, Justicia H, Silvia RM, Sonnenschein C. An "in culture" bioassay to assess the estrogenicity of xenobiotics (E-SCREEN). In: Chemically Induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection (Colborn T, Clement C, eds). Princeton, NJ:Princeton Scientific Publishing, 1992;295–309.
16. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR. New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 82:1107–1112 (1990).
17. Gilbert J, Doré J-C, Bignon E, Pons M, Ojasoo T. Study of the effects of basic di- and tri-phenyl derivatives on malignant cell proliferation: an example of the application of correspondence factor analysis to structure–activity relationships (SAR). Quant Struct Act Relat 13:262–274 (1994).

18. Soto AM, Justicia H, Wray JW, Sonnenschein C. *p*-Nonyl-phenol: an estrogenic xenobiotic released from "modified" polystyrene. Environ Health Perspect 92:167–173 (1991).
19. Soto AM, Sonnenschein C, Chung KL, Fernández MF, Olea N, Olea Serrano F. The E-SREEN as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ Health Perspect 103(suppl 7):113–122 (1995).
20. Leclercq G. Estrogens, antiestrogens and other estrogen compounds. In: Antitumor steroids (Blickenstaff RT, ed). New York:Academic Press, 1992;11–64.
21. Cummings AM, Metcalf JL. Methoxyclor regulates rat uterine estrogen induced protein. Toxicol Appl Pharmacol 130:154–160 (1995).
22. Mariotti A, Johnson S, Sodreholm K-JM. The estrogenic effect of bisGMA on estrogen-sensitive target tissues (abstract). J Dental Assoc 76:323 (1997).
23. Lewis DFV, Parker MG, King RJB. Molecular modelling of the human estrogen receptor and ligand interactions based on site-directed mutagenesis and amino acid sequence homology J Steroid Biochem Mol Biol 52:55–65 (1995).
24. Routledge EJ, Sumpter JP. Structural features of alkylphenolic chemicals associated with estrogenic activity. J Biol Chem 272:3280–3288 (1997).
25. Connor K, Rammamoorthy K, Moore M, Mustain M, Chen I, Safe S, Zacharewski T, Gillesby B, Joyeux A, Balaguer P. Hydroxylated polychlorinated biphenyls (PCBs) as estrogens and antiestrogens: structure–activity relationships. Toxicol Appl Pharmacol 145:111–123 (1997).
26. Knuppen R, Ball P, Emons G. Importance of A-ring substitution of estrogens for the physiology and pharmacology of reproduction. J Steroid Biochem 24:193–198 (1986).
27. Barral L, Cano J, López AJ, López J, Nogueira P, Ramírez C. Thermal degradation of a diglycidyl ether of bisphenol A/1,3-bisaminomethylcyclohexane (DGEBA/BAC) epoxy resin system. Thermochimica Acta 269:253–259 (1995).
28. Baker PJ. Polymers, polycarbonate. In: Encyclopedia of Chemical Processing and Design (McKelta JJ, ed). New York:Marcel Dekker, 1983;136–152.
29. Sharman M, Honeybone C, Jickels S, Castle L. Detection of residues of the epoxy adhesive component bisphenol A diglycidylether (BADGE) in microwave susceptors and its migration into food. Food Addit Contam 12:779–787 (1995).
30. Paseiro CP, Simal LJ, Paz AS, López MP, Simal GJ. Kinetics of the hydrolysis of bisphenol F diglycidyl ether in water-based food simulants. Comparison with bisphenol A diglycidyl ether. J Agric Food Chem 40:868–872 (1992).
31. Bourne LB, Milner FJM, Alberman KB. Health problems of epoxy resins and amine curing agents. Br J Ind Med 16:81–97 (1959).
32. Joost van T, Roesyanto ID, Satyawan I. Occupational sensitization to epichlorhydrin and Bisphenol A during the manufacture of epoxy resin. Contact Dermatitis 22:125–126 (1990).
33. Jolanki R, Kanerva L, Estlander T, Tarvainen K, Kerkinen H, Henricks-Eckerman ML. Occupational dermatoses from epoxy resin compounds. Contact Dermatitis 23:172–183 (1990).
34. Steiner S, Hönger G, Sagelsdorff P. Molecular dosimetry of DNA adducts in C3H mice treated with bisphenol-A diglycidylether. Carcinogenesis 13:969–972 (1992).
35. Climie IJG, Hutson DH, Stoydin G. Metabolism of the epoxy resin component 2,2-bis[4-(2,3-epoxypropoxy) phenyl] propane, the diglycidyl ether of bisphenol A (DGEBPA) in the mouse. Part I. A comparison of the fate of a single dermal application and of a single oral dose of ^{14}C-DGEBPA in the mouse. Xenobiotica 11:391–399 (1981).
36. Climie IJG, Hutson DH, Stoydin G. Metabolism of the epoxy resin component 2,2-bis[4-(2,3-epoxy propoxy) phenyl] propane, the diglycidyl ether of bisphenol A (DGEBPA) in the mouse. Part II. Identification of metabolites in urine and faeces following a single oral dose of ^{14}C-DGEBPA. Xenobiotica 11:401–424 (1981).
37. Lobos JH, Leib TK, Su TM. Biodegradation of bisphenol A and other bisphenols by a Gram-negative aerobic bacterium. Appl Environ Microbiol 58:1823–1831 (1992).
38. Spivack J, Leib TK, Lobos JH. Novel pathway for bacterial metabolism of Bisphenol-A. J Biol Chem 269:7323–7329 (1994).
39. Steinmetz R, Brown NG, Allen DL, Bigsby RM, Ben-Johathan N. The environmental estrogen bisphenol A stimulates prolactin release *in vitro* and *in vivo*. Endocrinology 138:1780–1786 (1997).
40. Nagel SC, vom Saal FS, Thayer KA, Dhar MG, Boechler M, Welshons WV. Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative *in vivo* bioactivity of the xenoestrogens bisphenol A and octylphenol. Environ Health Perspect 105:70–76 (1997).
41. Feldman D. Editorial: Estrogens from plastic—Are we being exposed? Endocrinology 138:1777–1779 (1997).
42. Colerangle JB, Deodutta R. Profound effects of the weak environmental estrogen-like chemical bisphenol A on the growth of the mammary gland of Noble rats. J Steroid Biochem Mol Biol 60:153–160 (1997).



Excellence in Environmental Research

Environmental Health Perspectives publishes monographs on important environmental health topics and an annual review issue as supplements to the monthly journal. The upcoming June Supplement (Volume 106, Supplement 3) focuses on children's environmental health issues. Topics of asthma and cancer are highlighted from the perspective of the basic researcher as well as the policymaker.

For subscription information, see p. A 122 – A 123

For advertising information call 919-541-5466 or e-mail surak@niehs.nih.gov