

Estrogenicity of Resin-based Composites and Sealants Used in Dentistry

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We tested some resin-based composites used in dentistry for their estrogenic activity. A sealant based on bisphenol-A diglycidylether methacrylate (bis-GMA) increased cell yields, progesterone receptor expression, and pS2 secretion in human estrogen-target, serum-sensitive MCF7 breast cancer cells. Estrogenicity was due to bisphenol-A and bisphenol-A dimethacrylate, monomers found in the base paste of the dental sealant and identified by mass spectrometry. Samples of saliva from 18 subjects treated with 50 mg of a bis-GMA-based sealant applied on their molars were collected 1 hr before and after treatment. Bisphenol-A (range 90–931 µg) was identified only in saliva collected during a 1-hr period after treatment. The use of bis-GMA-based resins in dentistry, and particularly the use of sealants in children, appears to contribute to human exposure to xenoestrogens. *Key words:* bisphenol-A, composite resins and sealants, E-screen bioassay, restorative dentistry, xenoestrogens. *Environ Health Perspect* 104:298–305 (1996)

The impact of certain estrogenic xenobiotics on the development, health, and reproductive systems of wildlife has been clearly documented (1). As data accumulate, environmental xenobiotics are also being implicated in human infertility, genital tract malformations, and increased cancer rates in estrogen target tissues (2,3). In 1936, Dodds and Lawson reported the estrogenicity of some diphenyl compounds containing two hydroxyl groups in *para* positions (4). Reid and Wilson (5) subsequently confirmed the estrogenicity of 4,4'-dihydroxydiphenylmethane derivatives. One such derivative, bearing two methyl groups and known as bisphenol-A, is a major component of epoxy resins. Bisphenol-A was found to leach from autoclavable polycarbonate laboratory flasks (6). Recently, we demonstrated that food packed in lacquer-coated cans was active in a bioassay for estrogenicity; bisphenol-A released from the epoxy resin lining of the cans was identified as the estrogenic contaminant (7).

Bisphenol-A is a common ingredient in restorative materials used in dentistry. Since the 1960s, when bisphenol-A diglycidyl methacrylate (bis-GMA)-based restorative materials were first used in odontology, many studies have assessed the effects of resins on pulpal injury (8) and their cytotoxic properties (9–11). Little attention was paid, however, to the systemic health effects of these chemicals or their monomers (12,13). Some studies focused on the alkylating properties of the glycidaldehyde portion of bisphenol-A diglycidylether (BADGE) (14). For example, the formation of glycidaldehyde adducts in adenine residues was demonstrated in mice after cutaneous treatment with BADGE (15).

Other studies examined the mutagenic and carcinogenic properties of epoxy resin monomers, with contradictory results (15).

Resin-based composite restorative materials used in dentistry ("composites") consist of two major components: an organic resin matrix and an inorganic filler. Composites without inorganic fillers are known as "sealants." Tooth-colored restorative materials are increasingly used for preventive purposes, to replace missing tooth structures and to modify tooth color and contour. The resin matrix is initially present as a fluid monomer that is converted into a rigid polymer by a free radical-initiated reaction of addition. The polymerization reaction (curing) can be chemically initiated (autocuring) or photoinitiated using ultraviolet or visible light in the presence of a photoinitiator.

Because of the low degree of polymerization required by the monomers in composites, concerns have been expressed about the leaching of chemicals from unpolymerized material, which is rapidly released after curing (16). *In vitro* studies have shown that most of the unpolymerized monomers of bis-GMA had leached 24 hr after setting. In addition, cured composites placed in the oral cavity are attacked mechanically and chemically. Enzymatic hydrolysis of methacrylates, together with mechanical forces, contribute to the breakdown of composite resins (17), which are slowly and persistently degraded (18).

A significant portion of the uncured or decomposed material that is swallowed can be absorbed by the intestine. Climie et al. (19,20) studied the metabolic degradation of ¹⁴C-BADGE in mice after the oral

administration of a single dose and found that 90% of the radioactivity was eliminated in feces and urine during the first 3 days of the experiment. Interestingly, a small amount of BADGE (~ 5%) underwent oxidative dealkylation to yield glycidaldehyde (which has alkylating properties) and bisphenol-A, among other by-products (20). The systemic behavior of the diphenolic derivative is poorly known and needs further investigation.

The purpose of this research was to determine whether bis-GMA-based restorative resins and their components have estrogenic activity in an *in culture* assay. We demonstrate here that the sealant and resin components bisphenol-A and bisphenol-A dimethacrylate are estrogenic and may represent an additional source of xenoestrogen exposure in humans.

Methods

Cell line and culture conditions. MCF7 human breast cancer cells originally established by Soule and colleagues (21) were at passages 100–106 postcloning at the time of study. 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% CO₂/95% air under saturating humidity at 37°C.

Plasma-derived human serum and removal of sex steroids. Plasma-derived human serum was prepared from expired plasma by adding calcium chloride to a final concentration of 30 mM to facilitate clot formation. We removed sex steroids from serum by charcoal-dextran stripping (22). Briefly, a suspension of 5% charcoal (Norit

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A, Sigma Chemical Co., St. Louis, Missouri) 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 1000g 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 1000g for 20 min, and the supernatant was then filtered through a 0.20- μ m filter (Gelman Sciences, Ann Arbor, Michigan). Charcoal dextran-treated human serum (CDHuS) was stored at -20°C until needed.

Cell proliferation experiments. We used MCF7 cells in the E-screen test of estrogenicity according to a technique slightly modified (23) from that originally described by Soto et al. (24). Briefly, cells were trypsinized and plated in 24-well plates (Limbro, McLean, Virginia) at initial concentrations of 10,000 cells per well in 5% FBS in DME. Cells were allowed to attach for 24 hr, then the seeding medium was 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. (25). Briefly, cells were treated with cold 10% trichloroacetic 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, California) at 492 nm. We verified the linearity of the SRB assay by cell number before cell growth experiments.

Results are expressed as means \pm SDs. We normalized mean cell numbers from each experiment to the steroid-free control cultures to correct for differences in the initial seeding density. Differences between the xenoestrogen and estradiol-17 β groups were assessed by analysis of variance and the *a posteriori* Scheffé's test. A *p*-value \leq 0.05 was regarded as significant.

Estrogen and progesterone receptor measurements. MCF7 cells were seeded in T-25 flasks in 5% FBS-supplemented DME. On the following day, the medium was changed to 10% CDHuS-supplemented

phenol red-free DME medium, and estradiol-17 β or the chemicals to be tested were added. One group of cells received vehicle alone. After 72 hr, the culture medium was discarded and cells were frozen in liquid N₂. To extract receptor molecules, we incubated cells at 4°C for 30 min with 1 ml extraction buffer (0.5 M KCl, 10 mM potassium phosphate, 1.5 mM EDTA, and 1 mM monothio glycerol, pH 7.4), according to a technique previously described in detail (26). After centrifugation to pellet the cell debris, estrogen and progesterone receptors were measured in a 100- μ l aliquot of the supernatant by enzyme immunoassay using the Abbott ER and PgR enzyme immunoassay monoclonal kits (Abbott Diagnostic, Wiesbaden, Germany) according to the manufacturer's instructions.

Estrogen-induced cell type-specific proteins. We measured cathepsin-D and pS2 in the culture medium of MCF7 cells with the ELSA-CATH-D and ELSA-PS2 immunoradiometric assays (CIS Bio International, Gif-sur-Yvette, France). The culture medium was centrifuged at 1,200g for 10 min to eliminate floating and detached cells. Samples were kept frozen at -80°C until the assays were conducted.

Competitive binding assays. Cytosol from immature, female rat uteri was prepared at a protein concentration of approximately 2 mg/ml in phosphate buffer. Aliquots of this 105,000g supernatant were then incubated with various concentrations of bisphenol-A, bisphenol-A dimethacrylate, BADGE, bis-GMA, and 3 nM [³H]estradiol for 16 hr at 0–4°C. The free and bound fractions were separated with the charcoal-dextran technique. We calculated the relative binding ability (RBA) of each competitor as the ratio of the concentration of radioinert estradiol/competitor required to inhibit 50% of the specific [³H]estradiol binding, with the affinity of estradiol set at 100%.

Determination of components in resin-based restorative material. We analyzed four commercial bis-GMA-based composite resins using an HPLC technique. Aliquots of 50–100 mg of each sample were suspended in 1 ml ethanol and chromatographed using a Lichocart Merck S5 ODS column (20 \times 0.4 cm; Merck, Darmstadt, Germany) at a flow rate of 1 ml/min in a Perkin-Elmer 250 binary LC chromatograph with a Perkin-Elmer 235 diode array detector 235 equipped with a 20- μ l loop injector (Waters 717 Plus Autosampler). The gradient was: at time = 0 min, 100% phase A (acetonitrile-water, 1:1) and 0% phase B (acetonitrile); at time = 15 min, 100% phase B and 0% phase A; and at time = 17 min, 100% phase A and

0% phase B. The elution profile was monitored at 280 and 254 nm.

Determination of bisphenol-A and related compounds in saliva. For the clinical study, 18 healthy men and women (age range 18–25 years, mean age 20 years) were treated. The experiment was conducted in accordance with the Helsinki Declaration. A sealant was applied to 12 molars according to a standard technique that included visible light as the activator of the curing agents. Samples of whole saliva were collected by having subjects spit for 1 hr before and 1 hr after treatment into preweighed glass flasks containing 10 ml ethanol. The amount of commercial bis-GMA-based sealant used was approximately 50 mg per subject. After collection, saliva samples were centrifuged (1000g, 10 min) and passed through a 0.45- μ m pore filter. Aliquots of 20 μ l were chromatographed by HPLC as described above and by GC/MS. Saliva samples were also tested for estrogenicity in the MCF7 breast cancer assay.

Spectroscopy. Mass spectra of extracted composite resins, filtered saliva samples, and technical grade bisphenol-A, dimethacrylate of bisphenol-A, and BADGE were obtained in a mass spectrometry system (Fisison VG Platform-II, Manchester, UK) operating at an ion source temperature of 200°C in a Fisison GC 8000 SM Autospec chromatograph (Milan, Italy). A 15-m methyl silicon column (OV-P) was used at a flow rate of 1.2 ml/min, with helium as the carrier gas. The oven temperature was 80–320°C, with a graded increase of 10°C/min. Relative retention times of all the components analyzed were 16.2 min for bisphenol-A; 20.5 min for BADGE, and 21.2 min for bisphenol-A dimethacrylate.

Steroids and chemical compounds tested. Estradiol-17 β was obtained from Sigma. Bisphenol-A, bisphenol-A dimethacrylate, BADGE, and bis-GMA were obtained from Aldrich (Aldrich-Chemie, Albu, Germany). Chemicals were dissolved in ethanol to a final concentration of 1 mM and stored at -20°C.

The resin based-materials we tested were Tetric (Ivoclar-Vivadent, Schaan, Liechtenstein), Charisma (Kulzer, Wehrheim, Germany), Pekalux (Bayer, Germany), identified as composites 1,2 and 3, respectively, and two different batches of the sealant Delton (Ash/Dentsply, Konstanz, Germany). All were diluted in phenol red-free DME immediately before use in the culture assay. The final ethanol concentration in the culture medium did not exceed 0.1%. We obtained [2,4,6,7-³H]estradiol (103 Bq/mmol) from Amersham (Buckinghamshire, England).

Results

Estrogenic Activity of Resin-based Composites

The addition of estradiol-17 β to CDHuS-supplemented medium increased the numbers of MCF7 cells in culture. Maximum proliferative effect was obtained at estradiol-17 β concentrations of 10 pM and higher (Fig. 1A). Cell yields were sixfold greater than in control cultures after 6 days (mean \pm SD, 6.67 ± 1.21 ; $n = 15$ experiments). In the absence of estradiol-17 β , cells proliferated minimally.

Four bis-GMA-based composite resins were assayed before polymerization using the MCF7 proliferation test. Composites and sealants were prepared in ethanol at a concentration of 100 and 50 mg/ml, respectively, and assayed at a range of dilutions (1/100 to 1/10⁶). The cell yield obtained with 5 μ g/ml sealant sample was sixfold greater than in control cultures. The magnitude of this proliferative effect was similar to that obtained with estradiol-17 β . Cell toxicity was observed at concentrations of 50 μ g/ml and higher (Fig. 1B).

In contrast to the sealant sample, the three resin-based composites assayed did not induce MCF7 cell proliferation at a maximal concentration of 1 mg/ml. These commercial formulations contain a large proportion of inorganic filler particles (50–85% by weight of the composite). Stock suspensions were prepared without discarding the inorganic portion.

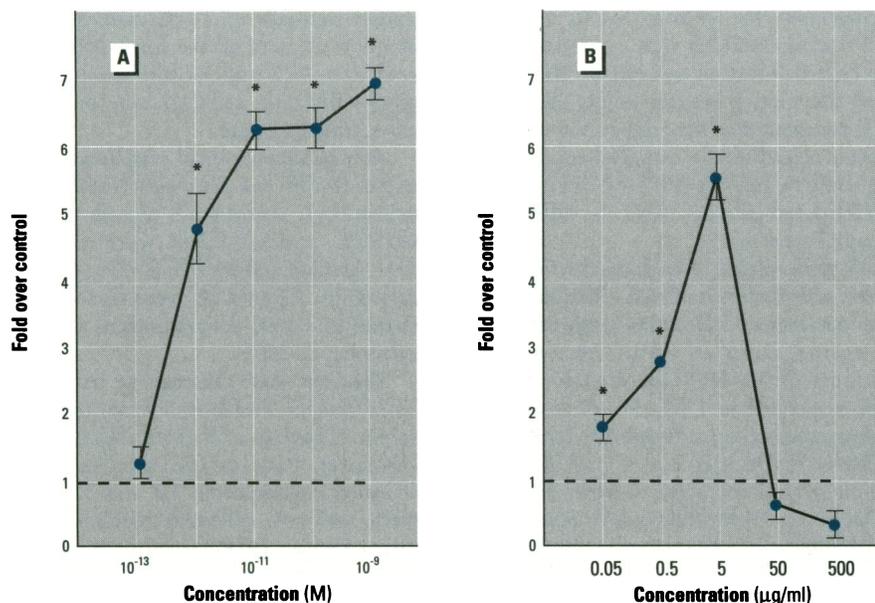


Figure 1. Cell proliferation of MCF7 cells. Cells growing in 10% charcoal dextran-treated human serum-supplemented medium were exposed for 144 hr to (A) estradiol-17 β and (B) bis-GMA-based sealant, sample no. 1. Points represent quadruplicate cultures; brackets indicate SDs. Ordinates represent cell numbers (experimental/control); abscissas represent concentration of the test compound. Estradiol-17 β was effective at ≥ 1 pM (0.272 pg/ml). *Values significantly different from control, $p < 0.001$.

Identification of Estrogenic Substances from Composite Resins

Figure 2 shows the HPLC profile of an unpolymerized sealant that was positive in the proliferation test. Relative retention times of the components identified were: $t = 2.35$ min or bisphenol-A; $t = 7.35$ min for BADGE; $t = 8.55$ min for bis-GMA; and $t = 11.85$ min for dimethacrylate of bisphenol-A. These components were quantified by calibration curves made after eluting known amounts (1 μ M to 1 mM) of the pure substance [bisphenol-A, $y = (2.92 \times 10^{11})x + (9.075 \times 10^6)$, $r = 0.998$; bis-GMA, $y = (1.04 \times 10^{11})x + (0.670 \times 10^6)$, $r = 0.999$; BADGE, $y = (1.12 \times 10^{11})x + (0.470 \times 10^6)$, $r = 0.999$, and bisphenol-A dimethacrylate, $y = (2.18 \times 10^{11})x + (8.782 \times 10^6)$, $r = 0.995$]. Mass spectrometric analysis confirmed the presence of bisphenol-A and dimethacrylate of bisphenol-A in all samples for which HPLC chromatograms showed the corresponding peaks (Fig. 3).

To determine whether the monomer bisphenol-A can be cleaved from composites and from their main oligomers (dimethacrylate of bisphenol-A, bis-GMA, and BADGE), these were hydrolyzed in alkaline (pH = 13) and acidic media (pH = 1) after heating (100°C for 30 min), and then chromatographed as indicated. Table 1 summarizes the components identified in the chromatograms. Extensive breakdown of the oligomers into smaller compounds such as bisphenol-A was observed.

Estrogenic Activity of Some Components of Resin-based Restorative Composites

Bisphenol-A and its dimethacrylate were estrogenic when assayed in the breast cancer cell proliferation assay. The concentrations required to produce maximum proliferation of MCF7 cells were 10,000-fold higher than those of estradiol-17 β (Fig. 4). MCF7 cells were grown in the presence of bisphenol-A or bisphenol-A dimethacrylate and the estrogen antagonist hydroxytamoxifen; the proliferative effect of 0.1 μ M bisphenol-A and bisphenol-A dimethacrylate disappeared when cells were grown in the presence of a 10-fold higher concentration of the antagonist (data not shown).

Bis-GMA was negative in the estrogenicity test. A proliferative effect of BADGE was observed at a concentration of 10 μ M (Fig. 5). Oligomers from both compounds became active in the proliferative test after hydrolysis (100°C for 30 min, in alkaline or acidic medium). Bisphenol-A and bisphenol-A dimethacrylate were identified in chromatograms when oligomers were found to be estrogenic after hydrolysis.

Progesterone Receptor Induction

MCF7 cells have receptors for estradiol-17 β (estrogen receptor; ER) and progesterone (Pgr). The basal concentration of

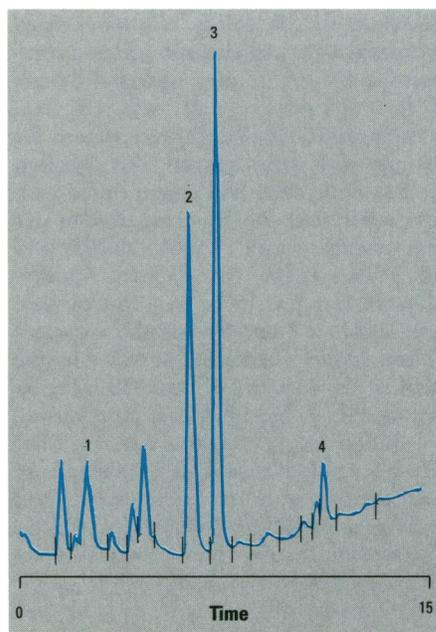


Figure 2. Chromatogram of sealant sample no. 1, which was estrogenic in the breast cancer proliferation bioassay shown in Figure 1. Peaks at retention times 2.3, 7.3, 8.6, and 11.9 min were identified as 1) bisphenol-A, 2) bis-GMA, 3) BADGE, and 4) bisphenol-A dimethacrylate.

ER in these cells was 183 ± 29 fmol/mg of extracted protein (Fig. 6A). Estradiol-17 β decreased the concentration of ER by 50% and increased that of PgR. The basal PgR value was 15.5 ± 4.3 fmol/mg protein, close to the lower limit of detection of the monoclonal antibody assay (Fig. 6B). Estradiol-17 β (1 nM) significantly increased PgR nearly 15-fold over the control value.

Treatment of MCF7 cells with concentrations of 0.1 μ M and higher of bisphenol-A dimethacrylate resulted in a significant increase in PgR levels. Bisphenol-A treatment also increased PgR levels (Fig. 6B). However, no reduction of ER levels was observed when bisphenol-A or bisphenol-A dimethacrylate induced PgR.

Secretion of pS2 and Cathepsin-D

Cathepsin-D and pS2 accumulation in the culture medium reflected increases in cell number during the experiments. The secretion of pS2 by MCF7 cells was significantly increased by ≥ 0.1 nM estradiol-17 β (~3.5-fold increase over controls). The basal concentration of pS2 (55.4 ± 15.2 ng/ 10^6 cells) increased to 202.2 ± 19.1 ng/ 10^6 cells after treatment with 10 μ M bisphenol-A dimethacrylate; this was similar to the effect of estradiol-17 β on pS2 secretion (Fig. 7A).

The basal concentration of cathepsin-D secreted by MCF7 cells was 8.9 ± 2.9 pmol/ 10^6 cells. Estradiol-17 β treatment (1 and 10 nM) resulted in a modest 1.7-fold increase in the accumulation of cathepsin-D in the culture medium. Bisphenol-A dimethacrylate (1 and 10 μ M) increased cathepsin-D secretion only 1.6-fold (Fig. 7B). These changes were not significant.

Binding Affinity of Composite Resin Compounds for the Estrogen Receptor

The binding affinity of bisphenol-A, bisphenol-A dimethacrylate, BADGE, and bis-GMA for the cytosol ER was determined by competitive binding analysis. The receptor relative binding affinities (RBA) were 0.012 and 0.0033 for bisphenol-A and bisphenol-A dimethacrylate, respectively, versus 100 for estradiol-17 β . Bis-GMA and BADGE did not compete with estradiol for binding to the ER, even at concentrations a million-fold higher than estradiol-17 β (Fig. 8).

Analysis and Quantification of Estrogenic Substances in Saliva

Eighteen saliva samples taken before and after sealant treatment were chromatographed using the technique described in Methods. Figure 9 shows the chromatograms.

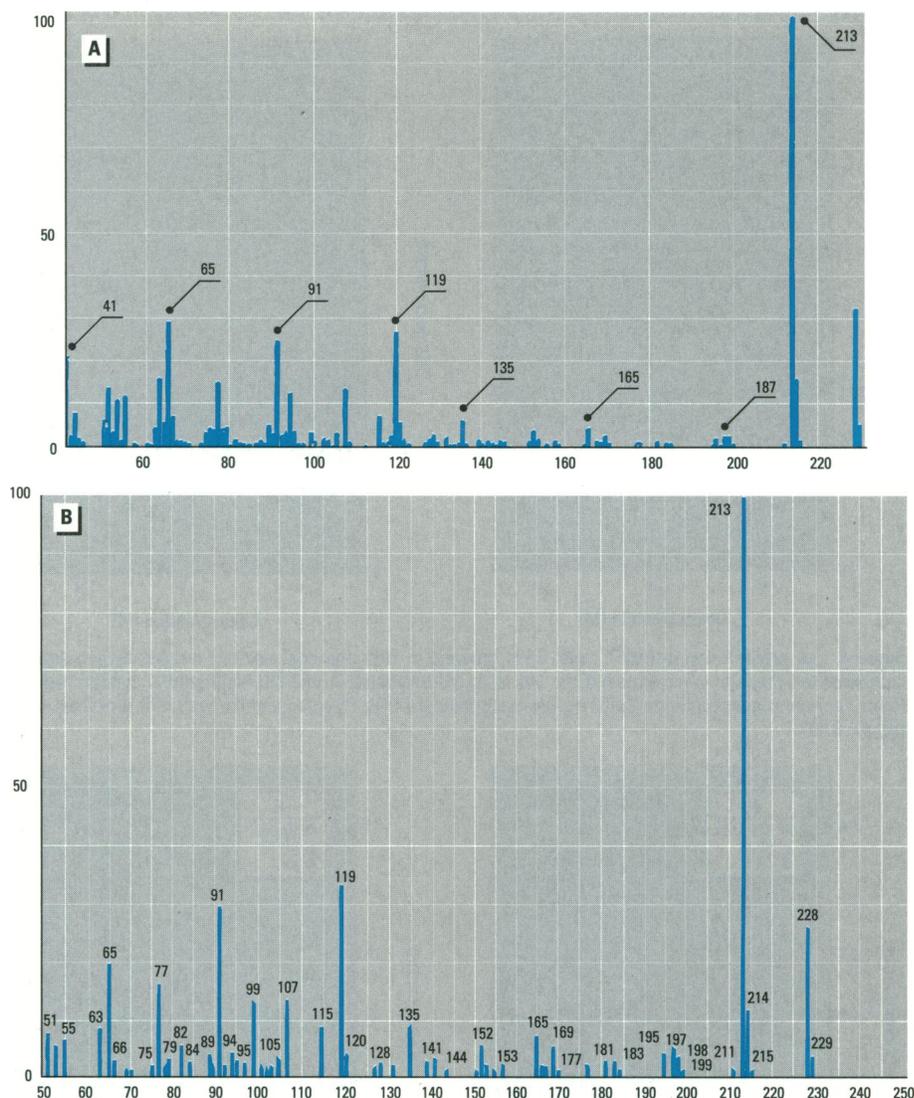


Figure 3. Mass spectra of (A) pure grade bisphenol-A and (B) bisphenol-A from a sealant sample.

Table 1. Identification of the components in the composites and sealants^a

Product and pH	μ g/100 mg commercial product			
	Bis-GMA	BADGE	Bisphenol-A dimethacrylate	Bisphenol-A
Sealant 1				
7	45.8	75.0	29.2	7.4
13	32.8	117.0	4.6	84.4
1	49.4	164.8	6.4	18.2
Composite 1				
7	105.0	19.6	ND	67.7
13	16.7	1.7	186.0	53.1
1	0.4	ND	1.8	49.9
Composite 2				
7	18.1	10.0	ND	0.5
13	53.2	24.2	878.4	ND
1	46.8	140.0	ND	ND
Composite 3				
7	22.0	3.2	ND	4.2
13	1809.4	38.6	ND	ND
1	1802.0	5.2	3.6	11.4

Abbreviations: bis-GMA, bisphenol-A diglycidylether methacrylate; BADGE, bisphenol-A diglycidylether; ND, not detectable.

^a Samples were hydrolyzed in alkaline (pH = 13) and acidic media (pH = 1) after heating (100°C for 30 min) and then chromatographed as indicated in Methods. The contents before hydrolysis are indicated as pH = 7.

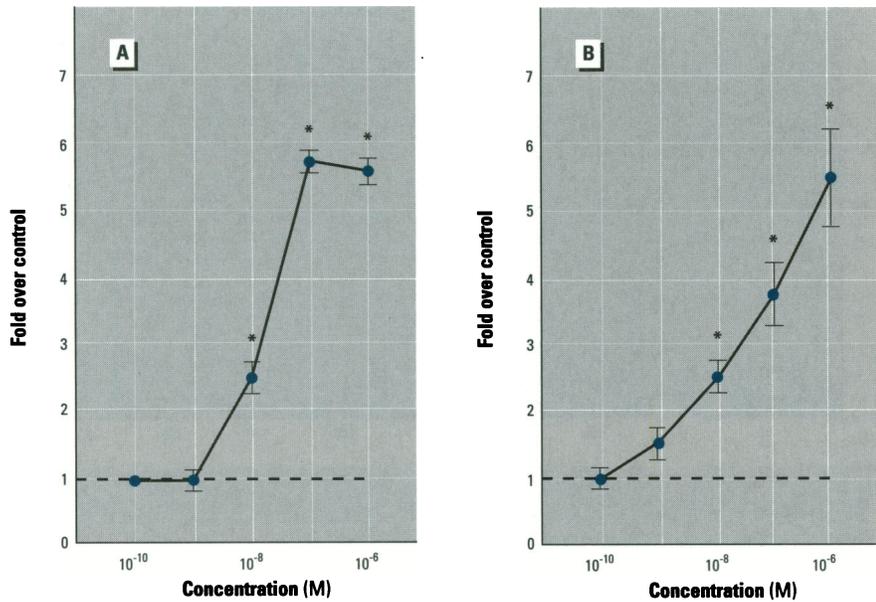


Figure 4. Cell proliferation in MCF7 cells. Cells growing in 10% charcoal dextran-treated human serum-supplemented medium were exposed for 144 hr to (A) bisphenol-A and (B) bisphenol-A dimethacrylate. Points represent quadruplicate cultures; brackets indicate SDs. *Values significantly different from control, $p < 0.001$.

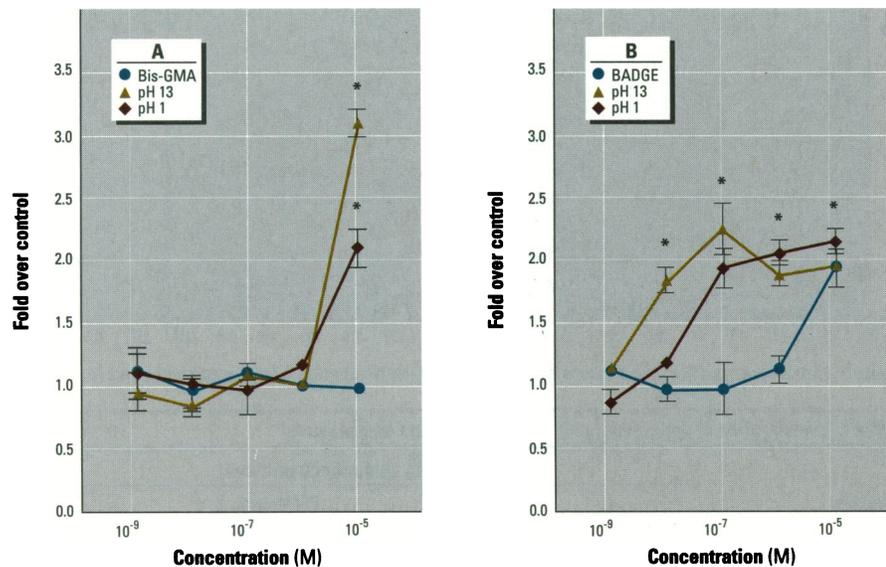


Figure 5. Cell proliferation in MCF7 cells. Cells growing in 10% charcoal dextran-treated human serum-supplemented medium were exposed for 144 hr to (A) bis-GMA and (B) BADGE and to products obtained by hydrolytic treatments of these compounds in alkaline (pH = 13) and acidic medium (pH = 1). Points represent quadruplicate cultures; brackets indicate SDs. *Values significantly different from control, $p < 0.001$.

graphic profile of a representative saliva sample from a patient selected at random after treatment with 50 mg of the bis-GMA-based sealant. Peaks in the chromatogram corresponded to Bis-GMA, BADGE, bisphenol-A, and dimethacrylate of bisphenol-A. Variations in the chromatographic profiles were seen between sealant batches and between patients who received sealant from the same batch. Monomers of composite components were

measured by HPLC in saliva collected 1 hr after treatment (Table 2). After treatment, all saliva samples contained variable amounts of bisphenol-A ranging from 90 to 931 μg ; this was confirmed by mass spectrometry.

Subjects were treated with a fixed amount of approximately 50 mg of sealant, and the proportion of free, unpolymerized material collected during 1 hr after treatment never exceeded 2% of the total

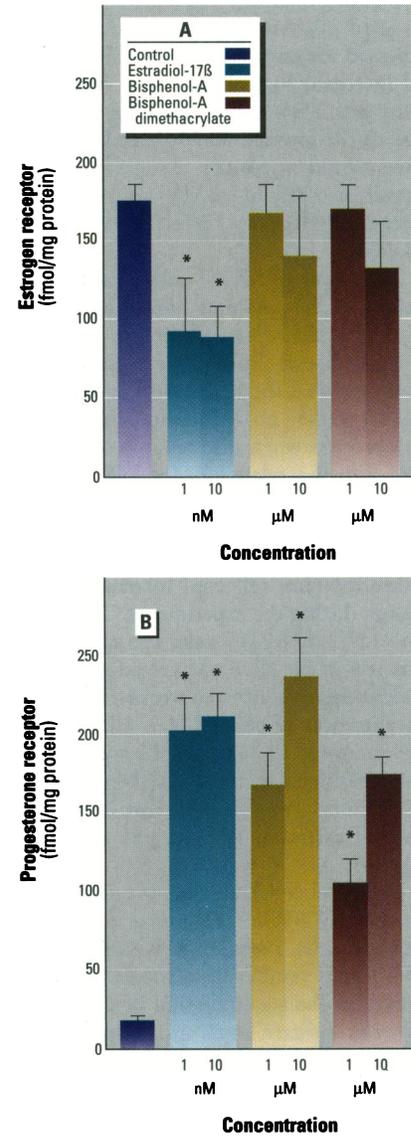


Figure 6. (A) Estrogen (ER) and (B) progesterone (PgR) receptors in MCF7 cells. Cells in T25 flasks were incubated in 10% charcoal dextran-treated human serum for 72 hr with 1 and 10 nM of estradiol-17 β . A parallel set of flasks was exposed to 1 and 10 μM bisphenol-A or bisphenol-A dimethacrylate. Controls received the vehicle alone. At the end of the experiment, the medium was aspirated and flasks were kept in liquid N₂ until assayed. ER and PgR were measured in extracted cells with a monoclonal antibody technique as indicated in Methods. Results are expressed as femtomole per milligram of extracted protein \pm SD. *Values significantly different from control, $p < 0.001$.

amount of sealant applied. Composite components were not observed in any of the saliva samples collected before treatment. A subject initially selected for treatment had been treated with tooth sealant 2 years earlier; chromatograms demonstrated the presence of bisphenol-A (66.4 μg) and bisphenol-A dimethacrylate (49.2 μg) in

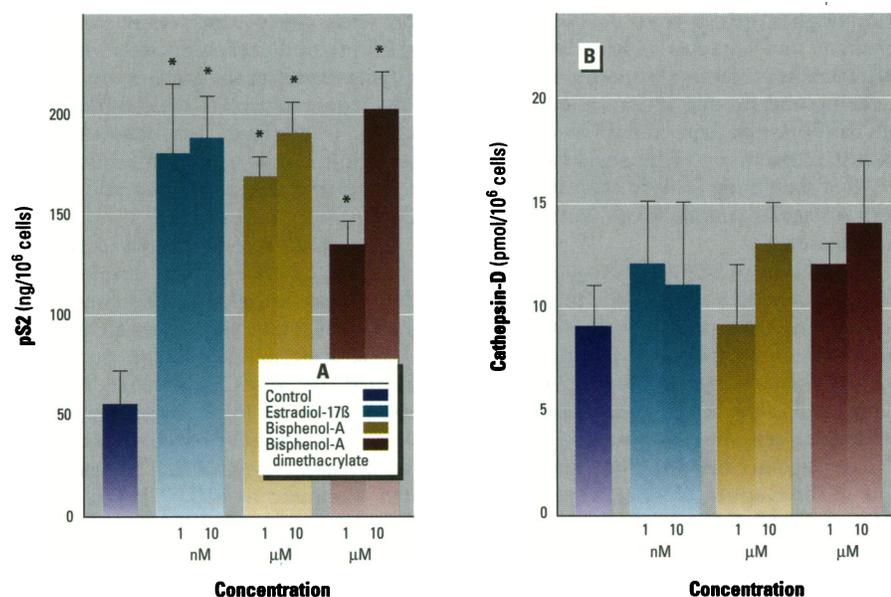


Figure 7. Accumulation of (A) pS2 and (B) cathepsin-D in culture medium. MCF7 cells were grown in 10% charcoal dextran-treated human serum-supplemented medium and exposed for 144 hr to 1 and 10 nM estradiol-17 β . Parallel cultures were exposed to 1 and 10 μ M bisphenol-A or bisphenol-A dimethacrylate. Controls received the vehicle alone. *Values significantly different from control, $p < 0.001$.

her saliva before the second treatment. The results from this subject were excluded from analysis.

Samples containing the highest amounts of bisphenol-A and bisphenol-A dimethacrylate were estrogenic in the proliferation test. Figure 10 shows the proliferative pattern of a sample containing 231 μ g bisphenol-A in 27 ml of collected saliva plus 10 ml of ethanol. An aliquot of 100 μ l of this sample diluted in 1 ml of the culture medium was positive in the estrogenicity assay. The estimated amount of bisphenol-A assayed was 0.62 μ g/ml. Because the presence of endogenous estrogens could not be ruled out, saliva samples taken before the sealant was applied were also assayed; none showed estrogenicity.

Discussion

Epoxy resins of the bisphenol-A type have many applications such as lacquer coating in food cans, in dental, surgical, and prosthetic devices, and as additives in a variety of other plastic materials (27). Here we demonstrate that bisphenol-A and bisphenol-A dimethacrylate, components of commercial resin-based composites and sealants used in dentistry, are estrogenic and may represent additional sources of human exposure to xenoestrogens.

Commercial smooth-surface composites vary in monomer composition and thus have different physical properties and biological behaviors. We found that the amount of bisphenol-A and bisphenol-A dimethacrylate varied between commercial

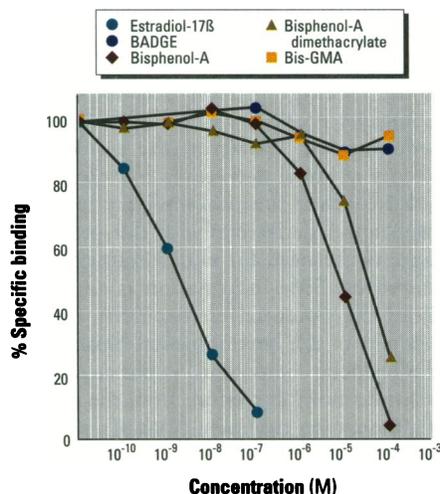


Figure 8. Competitive displacement of [3 H]-estradiol from the estrogen receptor by estradiol-17 β and bisphenol-A, bisphenol-A dimethacrylate, BADGE, and bis-GMA. The relative binding affinities of bisphenol-A and bisphenol-A dimethacrylate were 0.012 and 0.0033, respectively. [3 H]Estradiol concentration was 3 nM, and the total binding was 73 fmol/mg protein; $K_d = 8.5 \times 10^{-11}$ M.

composites and batches. Bisphenol-A was identified in all the composite samples and batches analyzed, whereas methacrylate of bisphenol-A was identified only in the sealant. The oligomers BADGE and bis-GMA were found in all three composites and in the sealant. The proportion of these compounds varied widely between composites and batches. Vankerckhoven et al. (28) studied 12 commercial restorative bis-

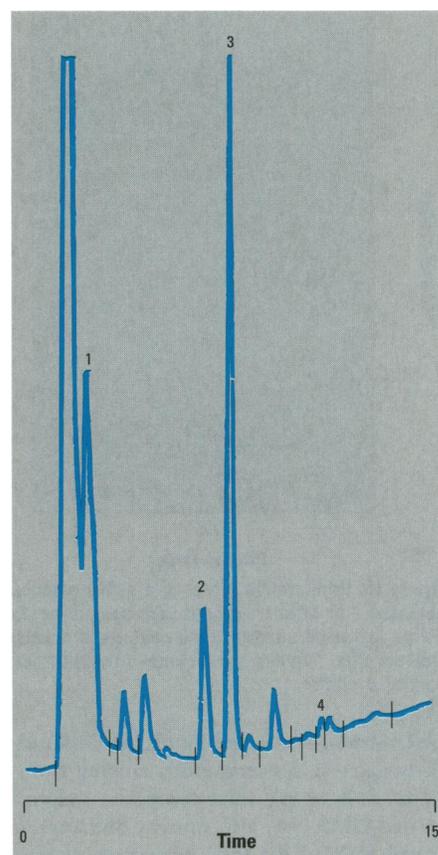


Figure 9. Chromatogram of a saliva sample with estrogenic activity. Peaks detected at retention times 2.3, 7.4, 8.5, and 11.9 min were identified as 1) bisphenol-A, 2) BADGE, 3) bis-GMA, and 4) bisphenol-A dimethacrylate, respectively.

Table 2. Quantitative evaluation of bisphenol-A identified in saliva obtained 1 hr after the application of 50 mg of sealant

Sample no	Sex	Volume (ml)	Bisphenol-A ^a	
			(μ g)	(μ g/ml)
1	F	29	320.6	11.1
2	F	31	931.0	30.0
3	F	35	174.6	4.9
4	F	33	865.6	26.2
5	M	26	91.2	3.5
6	M	31	249.6	8.1
7	F	27	165.7	6.1
8	F	38	263.6	6.9
9	M	32	105.3	3.3
10	M	27	230.5	8.5
11	M	36	181.4	5.0
12	M	35	324.2	9.3
13	M	29	353.0	12.2
14	M	39	810.8	20.8
15	M	32	230.8	7.2
16	M	27	130.4	4.8
17	M	27	89.8	3.3
18	F	29	112.4	3.9

^aSaliva samples were collected in glass flasks containing 10 ml ethanol. Values of bisphenol-A concentration represent those of undiluted saliva.

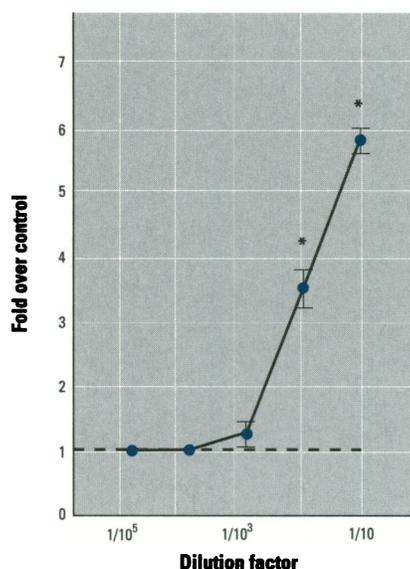


Figure 10. Proliferative effect of a saliva sample obtained 1 hr after treatment with sealant no. 1. Points represent quadruplicate cultures; brackets indicate SDs. *Values significantly different from control, $p < 0.001$.

GMA-based resins and found bis-GMA in all samples at concentrations ranging from 19 to 51% of the total composite weight. Iso-bis-GMA was also present and represented 40% of the total percentage of bis-GMA. Bisphenol-A dimethacrylate was found in four commercial mixtures in amounts that ranged from 6 to 10% of the total composite weight. In addition to these monomers, other compounds such as light activators, phthalates, and benzoates, as well as a large amount of inorganic fillers, are common ingredients of composites (28).

Once applied to tooth cavities, composites and sealants are polymerized *in situ*. The degree of conversion of oligomers into polymers varies depending on the composition of the resin and its distance from the tooth surface. Conversion of 60–75% is expected with most common composites (16), although levels of curing as low as 30% are found in the bottom of fillings. Lower levels of conversion are thought to be associated with greater elution of free components from the composites (16). A strong inverse relationship has been found between the leaching of resin from bis-GMA-based composites and monomer conversion. Monomer leaching reached a plateau at conversion levels of 60% (29). In nine commercial resins, the amount of residual bis-GMA measured at the end of the setting time was in the range of 0.4–1.2% of the original weight after curing. Amounts of the residual monomers of up to one-tenth the initial release were eluted into water over a period of 14 days

(29). Higher values were described by Ferracane and Condon (16), who found differences in percentage elution in relation to curing and double illumination. Our data confirm these figures: residual bisphenol-A in saliva after curing ranged from 0.1 to 2% of the 50 mg initially applied to the tooth surface in samples taken during 1 hr after treatment.

In addition to bisphenol-A, present in 100% of the saliva samples, its methacrylate derivative was present in 3 out of 18 samples. Both compounds were identified by chromatography and estrogenic activity as measured in the MCF7 breast cancer cell proliferation test, the E-screen assay.

The oligomer bis-GMA was not estrogenic in the E-screen bioassay. Hydrolyzed BADGE and bis-GMA in alkaline or acidic media were estrogenic. Chromatographic analyses of hydrolyzed samples detected the presence of bisphenol-A and bisphenol-A dimethacrylate after treatment. The metabolic routes of systemic degradation of some composite resins have been studied in mice (15,19,20). For example, BADGE is metabolized by oxidative dealkylation to give a phenol diol derivative. Much attention has been given to the smaller fragment of the cleavage process because of the mutagenic properties of the glycidylaldehyde portion. However, the estrogenic activity of these derivatives has not been evaluated; their chemical similarity to some bisphenol-A derivatives with this property suggests the need for closer examination. Moreover, little information is available about the potential estrogenicity of by-products of the environmental breakdown of biphenolic compounds. Lobos et al. (30) isolated a novel bacterium from the sludge of a wastewater treatment plant of a plastics manufacturing facility; the organism was able to degrade bisphenol-A to 4-hydroxybenzoic acid and 4-hydroxyacetophenone (both monophenolic by-products) and to 2,2-bis(4-hydroxyphenyl)-1-propanol, and finally to 2,3-bis(4-hydroxyphenyl)-1,2-propanediol. So far, the estrogenicity of these bisphenol-A derivatives has not been explored, although the similarity of the latter compound to some stilbenes suggests that it may display considerable activity.

Bisphenol-A is toxic to fish and invertebrates at concentrations of 1.1–10 mg/l ($\geq 4 \mu\text{mol/l}$) (31). In a mouse model, bisphenol-A induced photoallergic contact dermatitis (32). If administered during organogenesis, bisphenol-A is fetotoxic when administered to pregnant mothers. A study of the effects of bisphenol-A on reproduction and fertility showed a significant reduction in seminal vesicle weight and sperm motility in treated male mice

(33). These effects were present in parents and in a second generation when offspring were maintained at the same exposure level as their parents (4.37–17.5 mg/kg body weight). In addition, bisphenol-A treatment resulted in a significant reduction in the number of litters per pair and in live pups per litter.

Composites were discovered to be toxic in humans because lesions developed in unlined cavities (34,35). The concentrations to which cells and tissues are exposed in humans are unknown (11), but it was estimated that seven components of composites, including bisphenol-A, were toxic in the 10–100 $\mu\text{mol/l}$ concentration range in an in culture assay (11). The sealant we tested was toxic to MCF7 breast cancer cells at concentrations $> 50 \mu\text{g/ml}$ ($\sim 200 \mu\text{mol/l}$ of bisphenol-A). Lower concentrations were not toxic but were fully estrogenic in the bioassay (Fig. 1B).

Human exposure to bisphenol-A and dimethacrylate of bisphenol-A has increased during the last 30 years because of the extensive use of bisphenol-A-based resins. The use of sealants as an effective therapy and preventive treatment for dental pits and fissures in adults and children has been endorsed by European and U.S. agencies (18,36,37). In view of the documented exposure to bis-GMA-based composites and sealants used in dental treatments for adults and children, the use of these xenoestrogens should be reevaluated.

REFERENCES

- Colborn T, Clement C, eds. Chemically induced alterations in sexual and functional development: the wildlife/human connection. Princeton, NJ:Princeton Scientific Publishing, 1992.
- Sharpe R, Skakkebaek N. Are estrogens involved in falling sperm counts and disorders of the male reproductive tract? *Lancet* 341:1392–1395 (1993).
- Wolff MP, Toniolo P, Lee E, Rivera M, Dubin N. Blood levels of organochlorine residues and risk of breast cancer. *J Natl Cancer Inst* 85:648–652 (1993).
- Dodds EC, Lawson W. Synthetic estrogenic agents without the phenanthrene nucleus. *Nature* 137:996 (1936).
- Reid EE, Wilson E. The relation of estrogenic activity to structure in some 4,4'-dihydroxydiphenylmethanes. *J Am Chem Soc* 66:967–969 (1944).
- Krishnan AV, Starhis P, Permuth SF, Tokes L, Feldman D. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology* 132:2279–2286 (1993).
- Brotans 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).
- Hanks CT, Craig RG, Diehl ML, Pashley DH.

- Cytotoxicity in dental composites and other materials in a new *in vitro* device. *J Oral Pathol* 17:396–403 (1988).
9. Hensten-Petersen A, Helgeland K. Sensitivity of different human cell lines in the biologic evaluation of dental resin-based restorative materials. *Scand J Dent Res* 89:102–107 (1981).
 10. Terakado M, Yamazaki M, Tsujimoto Y, Kawashima T, Nagashima K, Ogawa J, Fujita Y, Sugiyama H, Sakai T, Furuyama S. Lipid peroxidation as a possible cause of benzoyl peroxide toxicity in rabbit dental pulp—a microsomal lipid peroxidation *in vitro*. *J Dent Res* 63:901–905 (1984).
 11. Hanks CT, Strawn SE, Wataha JC, Craig RG. Cytotoxic effects of resin components on cultured mammalian fibroblasts. *J Dent Res* 70:1450–1455 (1991).
 12. Bourne LB, Milner FJM, Alberman KB. Health problems of epoxy resins and amine curing agents *Br J Ind Med* 16:81–97 (1959).
 13. Morrissey RE, George JD, Price CJ, Tyl RW, Marr MC, Kimmel CA. The developmental toxicity of bisphenol A in rats and in mice. *Fundam Appl Toxicol* 8:571–582 (1987).
 14. Bentley P, Bieri P, Kuster H, Muakkassah-Kelly S, Sagelsdorff P, Staubli W, Waechter F. Hydrolysis of bisphenol A diglycidylether by epoxide hydro-lases in cytosolic and microsomal fractions of mouse liver and skin: inhibition by bis epoxy cyclopentylether and the effects upon the covalent binding to mouse skin DNA. *Carcinogenesis* 10:321–327 (1989).
 15. Steiner S., Hönger, Sagelsdorff P. Molecular dosimetry of DNA adducts in C3H mice treated with bisphenol A diglycidylether. *Carcinogenesis* 13:969–972 (1992).
 16. Ferracane JL, Condon JR. Rate of elution of leachable components from composite. *Dent Mater* 6:282–287 (1990).
 17. Freund M, Munksgaard EC. Enzymatic degradation of BISGMA/TEGDMA polymers causing decreased microhardness and greater wear *in vitro*. *Scan J Dent Res* 98:351–355 (1990).
 18. Ripa LW. Sealants revisited: an update of the effectiveness of pit-and-fissure sealants. *Caries Res* 27:77–82 (1993).
 19. 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 (DGEBA) in the mouse. Part I. A comparison of the fate of a single dermal application and of a single oral dose of ¹⁴C-DGEBA in the mouse. *Xenobiotica* 11:391–399 (1981).
 20. 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 (DGEBA) in the mouse. Part II. Identification of metabolites in urine and feces following a single oral dose of ¹⁴C-DGEBA. *Xenobiotica* 11:401–424 (1981).
 21. Soule HD, Vazquez J, Long A, Alberts S, Brennan MJ. A human cell line from a pleural effusion derived from a breast carcinoma. *J Natl Cancer Inst* 51:1409–1413 (1973).
 22. 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).
 23. Villalobos M, Olea N, Brotons JA, Olea MF, Ruiz de Almodóvar JM, Pedraza V. The E-screen assay: comparison among different MCF7 cell stocks. *Environ Health Perspect* 103:844–850 (1995).
 24. 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.
 25. 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).
 26. Maddedu L, Legros N, Devleeschouwer N, Bosman C, Piccart M, Leclercq G. Estrogen receptor status and estradiol sensitivity of MCF-7 cells in exponential growth phase. *Eur J Cancer Clin Oncol* 24:385–391 (1988).
 27. Paseiro Losada P, Simal Lozano J, Paz Abuin S, Lopez Mahia P, Simal Gándara J. Kinetics of the hydrolysis of bisphenol A diglycidyl ether (BADGE) on water-based food simulants. *Fres J Anal Chem* 345:527–532 (1993).
 28. Vankerckhoven H, Lambrechts P, Van Beylen M, Vanherle G. Characterization of composite resins by NMR and TEM. *J Dent Res* 60:1957–1065 (1981).
 29. Rueggeberg FA, Craig RG. Correlation of parameters used to estimate monomer conversion in a light-cured composite. *J Dent Res* 67:932–937 (1988).
 30. Lobos JH, Leib TK, Su T-M. Biodegradation of bisphenol A and other bisphenols by a gram-negative aerobic bacterium. *Appl Environ Microbiol* 58:1823–1831 (1992).
 31. Alexander HC, Dill DC, Smith LA, Guiney PA, Dorn P. Bisphenol A: acute aquatic toxicity. *Environ Toxicol Chem* 7:19–26 (1988).
 32. Maguire MC. Experimental photoallergic contact dermatitis to bisphenol A. *Acta Derm Venereol* 68:402–412 (1988).
 33. Dutch Expert Committee on Occupational Standards. Health-based recommended occupational exposure limit for bisphenol-A and its diglycidylether. Den Haag, The Netherlands:Committee of the Health Council of the Netherlands, 1995.
 34. Stanley HR, Bowen RL, Folio J. Compatibility of various materials with oral tissues. II Pulp responses to composite ingredients. *J Dent Res* 58:1507–1517 (1979).
 35. Inoue K, Hayashi I. Residual monomer (BIS-GMA) of composite resins. *J Oral Rehabil* 9:493–497 (1982).
 36. NIH. Seal out dental decay. Publication no. 91-489. Washington, DC:National Institutes of Health, 1991.
 37. Weintraub JA. The effectiveness of pit and fissure sealants. *J Public Health Dent* 49:317–330 (1989).