Xenoestrogens Released from Lacquer Coatings in Food Cans

José Antonio Brotons, María Fátima Olea-Serrano, Mercedes Villalobos, Vicente Pedraza, and Nicolás Olea

Laboratorio de Investigaciones Médicas, Universidad de Granada, 18071 Granada, Spain

We present data showing that some foods preserved in lacquer-coated cans and the liquid in them may acquire estrogenic activity. Hormonal activity was measured using the E-screen bioassay. The biological activity of vegetables packed in cans was a result of plastic monomers used in manufacturing the containers. The plastic monomer bisphenol-A, identified by mass spectrometry, was found as a contaminant not only in the liquid of the preserved vegetables but also in water autoclaved in the cans. The amount of bisphenol-A in the extracts accounted for all the hormonal activity measured. Although the presence of other xenoestrogens cannot be ruled out, it is apparent that all estrogenic activity in these cans was due to bisphenol-A leached from the lacquer coating. The use of plastic in food-packaging materials may require closer scrutiny to determine whether epoxy resins and polycarbonates contribute to human exposure to xenoestrogens. *Key words*: bisphenol-A, food containers, lacquer coating, xenoestrogens. *Environ Health Perspect* 103:608–612 (1995)

Epoxy resins are used as plastic coatings in the food-packing industry. It has been well documented that polymerization of epoxy resin reactions may not be fully complete, and that a significant proportion of unreacted epoxy compounds can be recovered from food packed in containers lined with these plastics (*1–3*). The migration of cured resin components into foods has also been reported. Unreacted epoxy compounds are thought to be toxic due to their alkylating properties (*4*).

A directive of the European Union (EU) has established a specific migration limit in food of 0.02 mg/kg for diglycidyl ether bisphenol-A (BADGE; CAS no. 1675-54-3). The presence of the monomer bisphenol A (4,4′-isopropopylidenediphenol, CAS no. 80-05-07) in these coatings was considered of lesser importance, and a higher tolerance limit (3 mg/kg) for its specific migration was therefore established by the EU Commission; however concerns about the toxicity of this compound were heightened recently when it was shown that bisphenol-A was estrogenic (*5*).

Europe Union directives 76/893, 80/590, 82/711, 85/572, and 90/128 summarize the European regulations of polymers in contact with foods. Specific migration can be assessed either in foods in contact with polymeric materials, or in substitutive simulants. Before testing, simulants should remain in contact with the interior of the can for similar periods and under similar conditions to those that characterize the product's normal shelf-life. For canned vegetables, the recommended simulants are distilled water or 3% acetic acid in water, depending on the pH of the preserved vegetables (water for pH >4.5, 3% acetic acid for pH <4.5). Moreover, for vegetables sterilized inside cans, testing polymer migration into simulants by heating cans at 121°C for 30–60 min was recommended.

The present study was designed to determine whether estrogenic activity due to plastic components was present in foods packed in lacquer-coated cans. Here we demonstrate the presence of estrogenic activity in foodstuffs inside cans and identify the estrogenic component as a chemical leached from the inner plastic coating.

Methods

Cell line and cell culture conditions. MCF7 human breast cancer cells originally established by Soule and colleagues (*6*) were a gift from C. Sonnenschein (Tufts University, Boston); they were at passages 70–103 after cloning at the time of study. For routine maintenance, cells were grown in our laboratory 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% $C0_2$/95% air under saturating humidity at 37°C.

Plasma-derived human serum and removal of sex steroids. 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 (*7*). Briefly, a suspension of 5% charcoal (Norit A; Sigma, 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 1000*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 4 cycles/min at 37°C for 1 hr. The suspension was centrifuged at 1000*g* for 20 min, and the supernatant was then filtered through a 0.20-µm filter (Gelman Sciences, Ann Arbor, Michigan). Charcoal dextran-treated human serum (CDHS) was stored at -20°C until needed.

Cell proliferation experiments in culture: E-screen test. MCF7 cells were used in the E-screen test according to a technique slightly modified from that originally described by Soto et al. (*8*). 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. The cells were allowed to attach for 24 hr; then the seeding medium was replaced with 10% CDHS supplemented phenol red-free DME. Different concentrations of the test compound were added. We stopped the assay after 144 hr by removing medium from wells, fixing the cells and staining them with sulforodamine-B (SRB). The staining technique was modified from that described by Skehan (*9*). Briefly, cells were treated with cold 10% trichloracetic acid (TCA) and incubated at 4°C for 30 min, then washed five times with tap water and left to dry. TCA-fixed cells were stained for 10 min with 0.4% (w/v) 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 evaluated linearity of the SRB assay with cell number before cell growth experiments. Alternatively, cells were lysed and the nuclei were counted with a Coulter ZM Counter apparatus (Coulter Electronics, Luton, England) according to a technique previously described in detail (*7*).

Results are expressed as means ±SD. Mean cell numbers from each experiment were normalized to the steroid-free control cultures to correct for differences in the initial seeding density. We assessed differences between the chemical compounds and estradiol-17ß groups using Student's *t*-test.

Extraction and determination of estrogenic compounds from lacquer-coated cans. Twenty different brands of canned foods were purchased in supermarkets in Spain and in the United States. Cans were packed in Brazil, France, Spain, Turkey and the United States.

The liquid from selected cans of whole green beans, artichoke hearts, asparagus

Address correspondence to N. Olea, Department of Radiology, Universidad de Granada, 18071 Granada, Spain.

We gratefully acknowledge A.M. Soto and C. Sonnenschein (Tufts University, Boston) for providing MCF7 cells and for helpful discussions and Karen Shashok for improving the English of the manuscript. This work was supported by grant 94/1551 from the Fondo de Investigaciones Sanitarias (FIS), Spanish Ministry of Health.

Received 31 October 1994; accepted 2 March 1995.

spears, corn, peas, mushrooms, palm hearts, peppers, tomatoes, and mixed vegetables was collected and filtered. A 75-mL aliquot was treated with 20 mL of methanol in a decantation funnel under agitation for 10 min. Samples were extracted with 40 mL chloroform and centrifuged at 1200*g* for 15 min; this process was repeated three times. Solvent was removed by concentration (1 mL) under reduced pressure at 60°C; 0.5 mL sulfuric acid was then added and the samples centrifuged. Finally, the organic phase was dried under a nitrogen stream.

Cans containing fatty foods such as sweetened condensed milk, condensed soup, pork and beans, concentrated milk-based infant formula, cheese dip, and tortillas were processed in a different way. Empty cans were filled up with bidistilled water (pH 7.5) and autoclaved for 30 min at 125°C with a total cycle time of 3 hr. The autoclaved water was processed like the liquid from vegetable and fruit cans.

Dried residues were suspended in 0.5 mL ethanol and chromatographed (Waters 501 HPLC System, Millipore, Milford, Massachusetts) using a Lichrocart Merck silica column (20 × 0.4 cm) (Merck, Darmstadt, Germany) at a flow rate of 1 mL/min with a 500 µL loop injector (Waters U6K). After 2 min of isocratic elution with *n*-hexane (Phase A), a gradient was applied from 0 to 40% phase B [*n*-hexane: methanol: isopropanol (40:45:15)], 10 min to 100% phase B, and 10 min to 100% phase A. The elution profile was monitored at 280 nm (Waters 490, Millipore). Fractions collected between 0 and 11 min (fraction α) and from 15 to 25 min (fraction ß) were pooled out, dried down, resuspended in 100 µL ethanol, and tested by the E-screen assay.

Alternatively, we quantified xenoestrogens in samples using a Perkin-Elmer 250 Binary LC with a Perkin-Elmer diode array detector and a Spherisorb silica S5 W column (25 × 0.4 cm) with a 20 µL loop injector (Rheodyne 7125, Perkin-Elmer). Working conditions were the same as described above.

Spectroscopic studies. Mass spectra of extracted cans and technical-grade bisphenol-A were obtained in a mass spectrometry system operating at an ion source temperature of 200°C in a Hewlett Packard 5890 chromatograph. A 30-m methyl silicon column (OV-P) was used with a 1.2 mL/min flow, and helium as the carrier gas. Temperature of the oven was 80–320°C, with a graded increase of 10°C/min.

Steroids and chemical compounds tested. Estradiol-17ß was obtained from Sigma (St. Louis, Missouri). Bisphenol-A was obtained from Aldrich (Albuch, Germany). Chemicals were dissolved in ethanol to a final concentration of 1 mM and stored at -20°C. They were all diluted in phenol red-free DME immediately before use. The final ethanol concentration in the culture medium did not exceed 0.1%.

Results

The addition of estradiol-17ß to CDHS-supplemented medium increased MCF7 cell numbers. Maximum proliferative effect was obtained with ≥10 pM estradiol-17ß (Fig. 1). The cell yield was sixfold greater than in control cultures (6.67 ± 1.21; n = 15 experiments). In the absence of estradiol-17ß, cells proliferated minimally.

Extracts from food packed in lacquer-coated cans were assayed with the E-screen test after chromatographic elution, as described in Methods. The proliferative effect eluted in the ß fraction significantly increased cell numbers in comparison with controls. Figure 2 shows cell yields of MCF7 cells supplemented with extracts from the liquid of peas packed in a lacquer-coated can. The cell yield for fraction ß was fourfold greater than in control. This proliferative effect was 58% of that obtained with estradiol-17ß. Extracts from

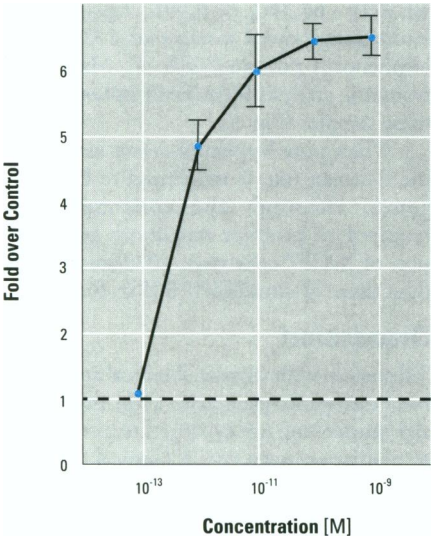

Figure 1. Cell proliferation in MCF7 cells. Cells growing in 10% charcoal dextran-treated human serum-supplemented medium were exposed for 144 hr to different amounts of estradiol-17ß. The points represent quadruplicate cultures; bars indicate standard deviations.

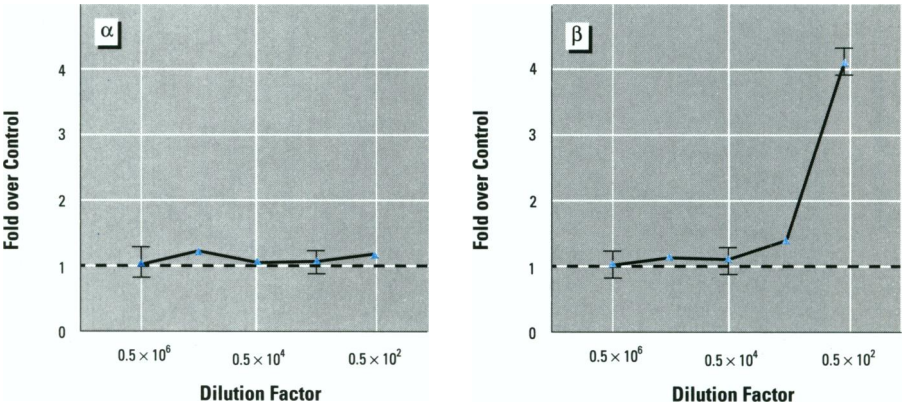

Figure 2. Cell proliferation in MCF7 cells. Cells growing in 10% charcoal dextran-treated human serum-supplemented medium were exposed for 144 hr to different dilutions of extracted liquid from food cans. Fractions α and β were tested. The proliferative effect eluted in the ß fraction significantly increased the cell numbers in comparison with controls. The points represent quadruplicate cultures; bars indicate standard deviations.

Table 1. Bisphenol-A concentration and estrogenic effects (means ± SD) of the liquid phase of vegetables packed in lacquer-coated cans

Vegetable	Can weight (g)	Amount of liquid (mL)	Bisphenol-A (µg/can)	Proliferative effect[a]
Peas	300	50	22.9 ± 8.8	3.9 ± 0.2*
Artichokes	390	150	18.6 ± 6.5	2.2 ± 0.1*
Green beans	400	190	11.9 ± 5.3	2.0 ± 0.2*
Mixed vegetables	450	220	10.1 ± 4.3	1.8 ± 0.2*
Corn	300	15	4.5 ± 2.6	1.5 ± 0.1*
Mushrooms	350	145	4.2 ± 4.1	1.7 ± 0.1*
Asparagus	230	80	ND	—
Palm hearts	500	280	ND	—
Peppers	390	140	ND	—
Tomatoes	390	140	ND	—
Estradiol-17ß (10 pM)	—	—	—	6.7 ± 1.2

ND, not detectable.
[a]Proliferative effect was estimated using 1/200 of the contents of each can.
*Significant difference from control cultures ($p < 0.01$).

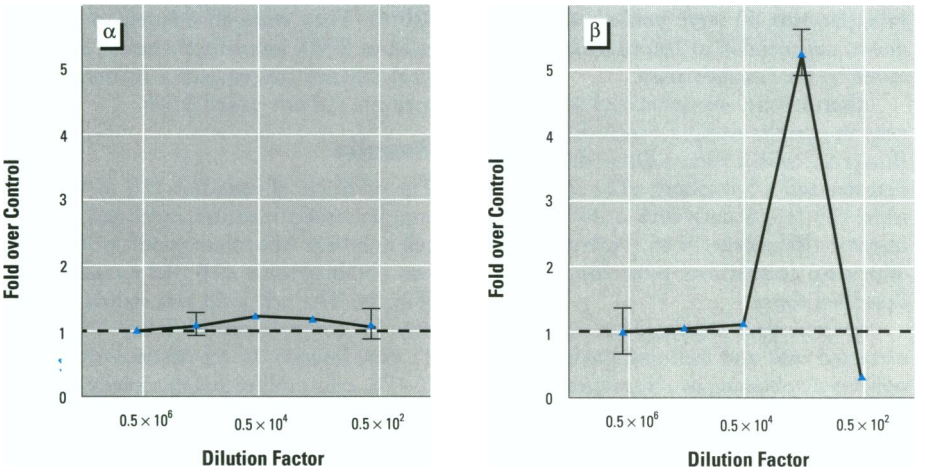

Figure 3. Cell proliferation in MCF7 cells. Cells growing in 10% charcoal dextran-treated human serum-supplemented medium were exposed for 144 hr to different dilutions of water from autoclaved cans. Fractions α and β were tested. The proliferative effect eluted in the ß fraction significantly increased the cell numbers in comparison with controls. The points represent quadruplicate cultures; bars indicate standard deviations.

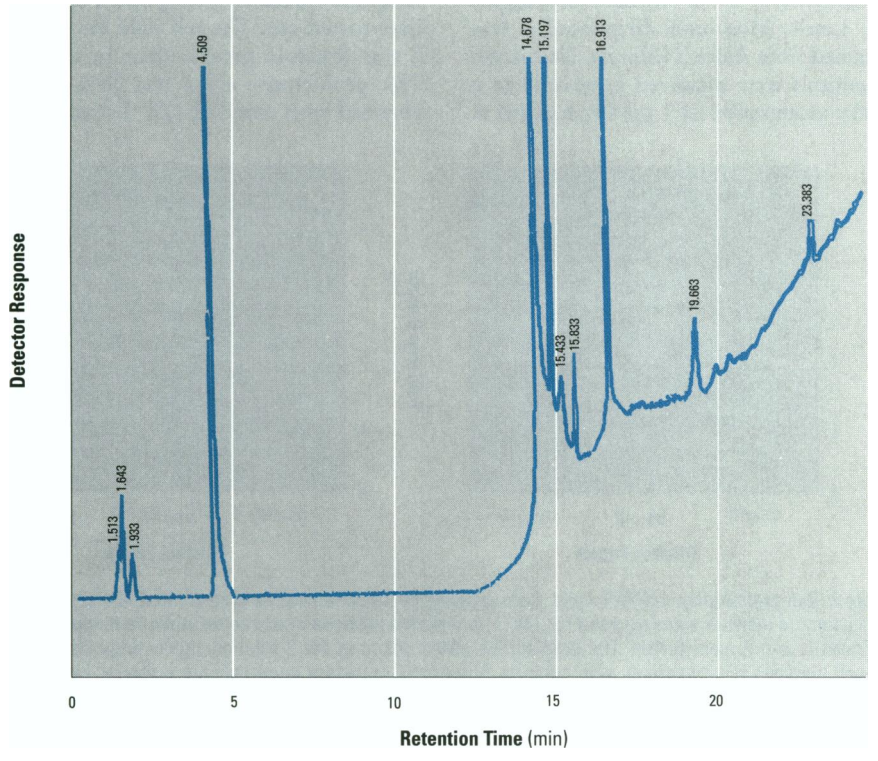

Figure 4. Chromatogram of extracts of lacquer-coated cans showing estrogenic effect in the E-screen bioassay. Fractions collected between 0 and 11 min (fraction α) were negative in the E-screen test, whereas fractions from 15 to 25 min (fraction ß) were estrogenic.

peas, artichoke hearts, corn, mushrooms, whole green beans, and mixed vegetables packed in lacquer-coated cans also showed estrogenic activity (Table 1).

We conducted several experiments to assess whether the estrogenic chemicals were leached from the walls of lacquer-coated cans during the autoclaving process or were released from the vegetables. First, empty cans containing water were autoclaved for 25 min at 125°C; the autoclaved water was processed and then tested with the E-screen assay. Second, fresh fruits and vegetables that were positive in the bioassay when packed in lacquer-coated cans were extracted and tested with the E-screen assay. No estrogenic activity was detected in fresh vegetables (results not shown). In contrast, water autoclaved in cans in which estrogenic activity was found also showed estrogenic activity (Fig. 3).

Some of the lacquer-coated cans that had contained fatty foods also released estrogenic chemicals into water after autoclaving. For example, extracts from cans that had contained sweetened condensed milk increased cell proliferation by as much as 70% of the maximal effect of estradiol-17ß.

Extraction efficiency was assayed with three solvents for bisphenol-A. Different amounts of bisphenol-A, ranging from 3.15 to 10 µg were extracted with hexane, hexane:ethyl ether (1:1 v/v), or chloroform. Hexane extraction failed to recover bisphenol-A; however, with hexane:ethyl ether, percentage recoveries were from 60.2 ± 5.6% to 63.1 ± 4.1%. Chloroform showed the highest extraction efficiency, which ranged from 88.4 ± 6.4% to 89.2 ± 4.5%. We chose chloroform to extract bisphenol-A from the liquid phase of canned foods and simulants.

Recovery rates were also tested before investigating bisphenol-A content in canned foods. The liquid from vegetables packed in glass containers was spiked with known amounts of bisphenol-A ranging from 10 to 70 µg and then extracted according the protocol described in Methods. Recovery rates varied from 82.4 ± 6.1% to 86.2 ± 4.3%.

The chromatographic profile of an extract of the liquid from a lacquer-coated can containing peas is shown in Figure 4. Among the peaks detected, bisphenol-A was identified as having a retention time of 19.69 min. This compound was present in some extracted foods and in water from autoclaved cans; the peak was present in all extracts with estrogenic activity, and all extracts having bisphenol-A were estrogenic. Bisphenol-A was quantified by a calibration curve made after eluting known amounts of the pure substance (y = 30,769x – 67,366; r = 0.998). Quantitative evaluation showed a range of 4–23 µg of bisphenol-A per can (Table 1). Mass spectrometric analysis identified bisphenol-A in these samples (Fig. 5).

When pure bisphenol-A was assayed in the E-screen test, it was found to be estrogenic, although the concentrations required to produce maximum proliferation of MCF7 cells were 1000-fold higher than those of estradiol-17ß (Fig. 6).

Discussion

Substances with diverse chemical structures have estrogenic properties (*10*); this diversity hinders the use of structure as the basis to predict estrogenicity. Chemical diversity thus represents a considerable setback to regulatory efforts aimed at safeguarding the public from the harmful effect of xenobiotics. Simple, reliable biological assays such as the E-screen test (*8*) have been proposed to remedy this situation. This bioassay is based on the ability of chemicals that are estrogenic to induce the proliferation of

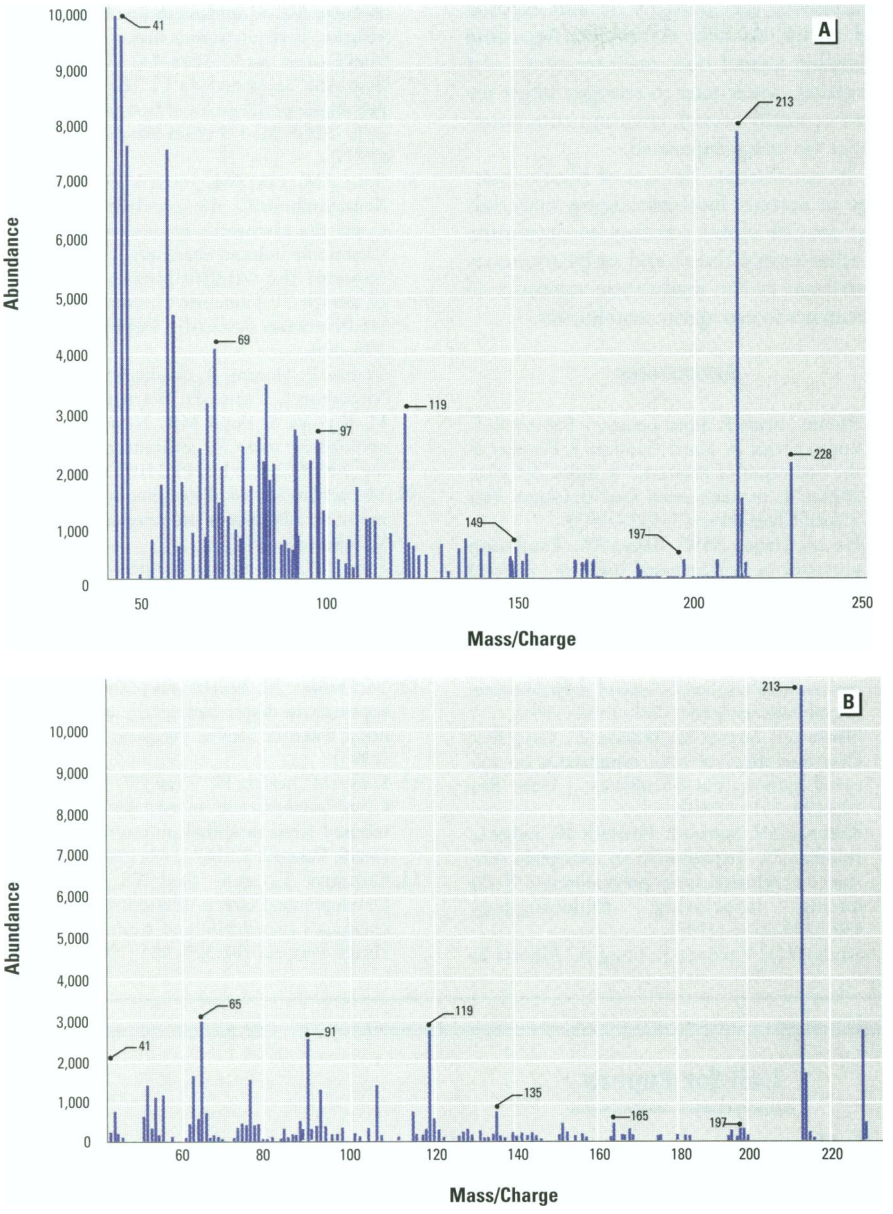

Figure 5. (A) Mass spectrum of extracted lacquer-coated cans positive in the E-screen bioassay. (B) Mass spectrum of bisphenol-A.

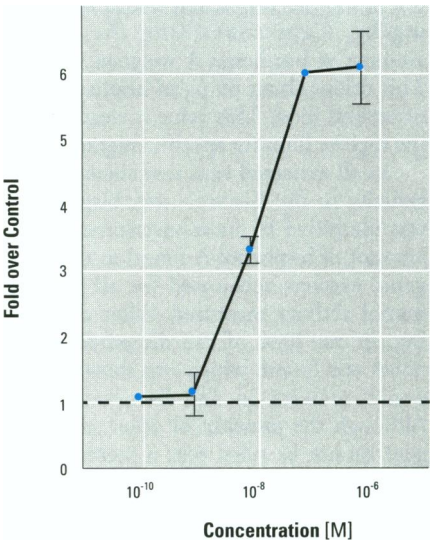

Figure 6. Cell proliferation in MCF7 cells. Cells growing in 10% charcoal dextran-treated human serum-supplemented medium were exposed for 144 hr to variable dilutions of bisphenol-A. The points represent quadruplicate cultures; bars indicate standard deviations.

cells of the MCF7 breast tumor cell line (*6*) under well-defined, reproducible conditions (*7*). The E-screen test has been credited with the identification of pesticides whose estrogenic properties were unsuspected (*8,11*), and it represents a valuable tool in environmental toxicology (*12*).

The data reported in this paper strongly suggest that some foods preserved in lacquer-coated cans acquire estrogenic activity. This biological activity may be related to estrogenic substances such as phytoestrogens or estrogenic pesticides contained in vegetables before they are canned, or plastic monomers or additives used in the manufacture of food containers. Phytoestrogens and organochlorine pesticides were not found in the foods packed in the cans we studied, nor were they detected in fresh vegetables. To test the second possibility, cans in which the vegetables were packed were filled with distilled water and autoclaved to determine whether this treatment released estrogenic components from the inner plastic coating. The plastic monomer bisphenol-A was found as a contaminant not only in the liquid of the preserved vegetables, but also in water autoclaved in these cans.

Monomers and oligomers may be released during the setting period, when conversion of the oligomer into a polymer is incomplete, and when polymerized resin is degraded by high temperatures, autoclaving, enzyme hydrolysis, etc. Bisphenol-A was reported to leach from polycarbonate tubes during autoclaving. Krishnan et al. (*5*) found concentrations of up to 15 mM of this compound (equivalent to 2.3–3.5 µg bisphenol-A/L water) in distilled water after autoclaving. Because acidic water (pH 5.5) was used, whereas hydrolysis of polycarbonate resins is favored by alkaline pH, the amount of bisphenol-A extracted in the study by Krishnan and colleagues may underestimate the amount of this chemical that is potentially extractable.

Foods packed in lacquer-coated cans are sterilized by autoclaving; thus the experiments described by Krishnan et al. (*5*) are to a substantial degree reproduced in the processing of canned food. The amount of bisphenol-A we extracted from the liquid of canned foods ranged from 0 to 33 µg per can. The higher concentrations are above the values found by Krishnan and colleagues. Food in the lacquer-coated cans we studied was in contact with the polymer for a longer time than was the water in flasks used the experiments by Krishnan et al. After cans are autoclaved by the manufacturer, they are usually stored for months before they are sold. This prolonged period may favor the accumulation of bisphenol-A. Leaching of bisphenol-A may also be related with the type of polymer, the sterilization procedure, and the type of food contained. Krishnan and colleagues do not state how many times they attempted to detect bisphenol-A from reautoclaved flasks; however, we found that this compound continued to be released from lacquer-coated cans after a second autoclaving.

The directive of the European Union establishes a specific limit of migration of

the monomer bisphenol-A into food of 3 mg/kg. Cans containing the highest amount of bisphenol-A weighed 0.30 kg. This means about 80 µg of bisphenol-A/kg of canned food. This value is clearly below the highest limit for specific migration.

In all extracted cans that showed estrogenicity in the E-screen test, bisphenol-A was identified by mass spectrometry. The amount of bisphenol-A found in the estrogenic extracts accounted for all the hormonal activity measured. After autoclaving, in one kind of can dimethyl bisphenol-A was found, which also showed estrogenic activity in the E-screen assay. Although the presence of other xenoestrogens cannot be ruled out, it seems reasonable to attribute all the estrogenic activity found in these cans to bisphenol-A leached from the lacquer coating. Studies are in progress to identify other resin components with estrogenic activity. Interestingly, we have found dimethacrylate of bisphenol-A, a component of composite resins used as restorative materials in dentistry, to be estrogenic in the E-screen assay at 10- to 100-fold lower concentrations than bisphenol-A (Pérez et al., manuscript in preparation). Alternative sources of these xenoestrogens such as laboratory contamination (*13*) were ruled out.

The impact of certain estrogenic xenobiotics on the reproductive system, development and health of animals has been clearly documented (*14*). Findings such as ours demonstrate that humans are also exposed and at risk. As data accumulate regarding infertility, genital tract malformations, and increasing cancer rates in estrogen target tissues (especially breast), environmental xenobiotics are being implicated.

In conclusion, the use of plastic coatings in certain food-packaging materials may require closer scrutiny to determine whether epoxy resins and polycarbonates contribute to the inadvertent exposure of consumers to estrogenic xenobiotics.

References

1. Paseiro Losada P, Simal Lozano J, Paz Abuin S, Lopez Mahia P, Simal Gandara J. Kinetics of the hydrolysis of bisphenol A diglycidyl ether (BADGE) in water-based food simulants. Fres Z Anal Chem 345:527–532 (1993).
2. He M, Urban MW, Bauer RS. Exudation processes in hydrogenated bisphenol-A-based epoxy coatings: spectroscopic study. J Appl Polymer Sci 49:345–359 (1993).
3. Rufus IB, Shah H, Hoyle CE. Identification of fluorescent products produced by the thermal treatment of bisphenol-A-based polycarbonate. J Appl Polymer Sci 51:1549–1558 (1994).
4. Hanks CT, Strawn SE, Wataha JC, Craig RG. Cytotoxic effects of resin components on cultured mammalian fibroblasts. J Dent Res 70:1450–1455 (1991).
5. 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).
6. 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).
7. 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).
8. Soto AM, Lin TM, Justicia H, Silvia RM, Sonnenschein C. An in culture bioassay to assess the estrogenicity of xenobiotics. In: Chemically induced alterations in sexual development: the wildlife/human connection (Colborn T, Clement C, eds). Princeton, NJ:Princeton Scientific Publishing, 1992; 295–309.
9. 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).
10. McLachlan JA, ed. Estrogens in the environment II: influences on development. New York:Elsevier, 1985.
11. Soto AM, Chung KL, Sonnenschein C. The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. Environ Health Perspect 102:380–383 (1994).
12. McLachlan JA. Functional toxicology: a new approach to detect biologically active xenobiotics. Environ Health Perspect 101:386–387 (1993).
13. 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).
14. Colborn T, vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101:378–384 (1993).