Commentary

An Extensive New Literature Concerning Low-Dose Effects of Bisphenol A Shows the Need for a New Risk Assessment

Frederick S. vom Saal[1] and Claude Hughes[2,3]

[1]Division of Biological Sciences, University of Missouri, Columbia, Missouri, USA; [2]Department of Medical and Scientific Services, Quintiles, Research Triangle Park, North Carolina, USA; [3]Department of Biology, East Carolina University, Greenville, North Carolina, USA

Bisphenol A (BPA) is the monomer used to manufacture polycarbonate plastic, the resin lining of cans, and other products, with global capacity in excess of 6.4 billion lb/year. Because the ester bonds in these BPA-based polymers are subject to hydrolysis, leaching of BPA has led to widespread human exposure. A recent report prepared by the Harvard Center for Risk Analysis and funded by the American Plastics Council concluded that evidence for low-dose effects of BPA is weak on the basis of a review of only 19 studies; the report was issued after a delay of 2.5 years. A current comprehensive review of the literature reveals that the opposite is true. As of December 2004, there were 115 published *in vivo* studies concerning low-dose effects of BPA, and 94 of these report significant effects. In 31 publications with vertebrate and invertebrate animals, significant effects occurred below the predicted "safe" or reference dose of 50 µg/kg/day BPA. An estrogenic mode of action of BPA is confirmed by *in vitro* experiments, which describe disruption of cell function at 10^{-12} M or 0.23 ppt. Nonetheless, chemical manufacturers continue to discount these published findings because no industry-funded studies have reported significant effects of low doses of BPA, although > 90% of government-funded studies have reported significant effects. Some industry-funded studies have ignored the results of positive controls, and many studies reporting no significant effects used a strain of rat that is inappropriate for the study of estrogenic responses. We propose that a new risk assessment for BPA is needed based on *a)* the extensive new literature reporting adverse effects in animals at doses below the current reference dose; *b)* the high rate of leaching of BPA from food and beverage containers, leading to widespread human exposure; *c)* reports that the median BPA level in human blood and tissues, including in human fetal blood, is higher than the level that causes adverse effects in mice; and *d)* recent epidemiologic evidence that BPA is related to disease in women. *Key words:* bisphenol A, dose response, endocrine disruptors, low dose, nonmonotonic, risk assessment scientific integrity. *Environ Health Perspect* 113:926–933 (2005). doi:10.1289/ehp.7713 available via *http://dx.doi.org/* [Online 13 April 2005]

Bisphenol A (BPA) is a known environmental estrogen that is used as the monomer to manufacture polycarbonate plastic, the resin that is used as linings for most food and beverage cans, as dental sealants, and as an additive in other widely used consumer products. BPA is one of the highest-volume chemicals produced worldwide; global BPA capacity in 2003 was 2,214,000 metric tons (> 6.4 billion lb), with 6–10% growth in demand expected per year (Burridge 2003). Heat and contact with either acidic or basic compounds accelerate hydrolysis of the ester bond linking BPA molecules in polycarbonate and resins. Specifically, heating of cans to sterilize food, the presence of acidic or basic food or beverages in cans or polycarbonate plastic, and repeated washing of polycarbonate products have all been shown to result in an increase in the rate of leaching of BPA (Brotons et al. 1995; Consumers Union 1999; Howdeshell et al. 2003; Kang and Kondo 2002; Kang et al. 2003; Olea et al. 1996; Raloff 1999). In addition, another potential source of human exposure is water used for drinking or bathing. Studies conducted in Japan (Kawagoshi et al. 2003) and in the United States (Coors et al. 2003) have shown that BPA accounts for most estrogenic activity that leaches from landfills into the surrounding ecosystem.

Convincing evidence that there is widespread exposure to BPA is shown by the finding of Calafat et al. (2005) that 95% of urine samples from people in the United States examined by the Centers for Disease Control and Prevention (CDC) have measurable BPA levels [range, 0.4 ppb (10th percentile) to 8 ppb (95th percentile); median = 1.3 ppb]. As described by Calafat et al. (2005), these levels are consistent with findings from other countries. For example, levels of unconjugated (parent) BPA in human blood and tissues are also in the same 0.1–10 ppb range (Ikezuki et al. 2002; Schonfelder et al. 2002) detected by Calafat et al. (2005) in urine. Because there is evidence that BPA is rapidly metabolized (Volkel et al. 2002), these finding suggest that human exposure to significant amounts of BPA must be continuous and via multiple sources. A relationship between blood levels of BPA and body fat in women has been reported (Takeuchi et al. 2004).

In this commentary, we document for the scientific, public health, and regulatory communities that exposure of experimental animals to "low doses" of BPA, which result in tissue levels within and even below the range of human exposure, has been related to adverse effects in a large number of recently published studies. A recent case–control study reporting that blood levels of BPA are related to ovarian disease in women (Takeuchi et al. 2004) adds to our concern. A large number of *in vitro* studies show that effects of BPA are mediated by both genomic and nongenomic estrogen-response mechanisms, with disruption of cell function occurring at doses as low as 1 pM or 0.23 ppt (Wozniak et al. 2005). Although the focus of most studies of effects of BPA has been on its estrogenic activity, recent reports indicating the potential to disrupt thyroid hormone action (Moriyama et al. 2002; Zoeller et al. 2005) mean other modes of action must also be considered. Very low part-per-trillion doses of BPA also cause proliferation of human prostate cancer cells via binding to a mutant form of the androgen receptor expressed in a subpopulation of prostate cancer cells (Wetherill et al. 2002), although BPA acts as an androgen antagonist in the presence of the wild-type androgen receptor (Lee et al. 2003; Paris et al. 2002) and can also block testosterone synthesis (Akingbemi et al. 2004). A comprehensive document containing all of the low-dose BPA references, as well as information concerning mechanisms of action, pharmacokinetics, sources of exposure, and exposure levels in humans, is available online (Endocrine Disruptors Group 2005).

Our current conclusion that widespread exposure to BPA poses a threat to human health directly contradicts several recent reports from individuals or groups associated with or funded by chemical corporations [Association of Plastics Manufacturers in

Europe (APM) 2005; Gray et al. 2004; Kamrin 2004; Purchase 2004]. For example, a recently published report on BPA prepared by a panel convened by the Harvard Center for Risk Analysis (HCRA), which was funded by the American Plastics Council (APC), concluded that "the weight of the evidence for low-dose effects is very weak" (Gray et al. 2004). However, the charge to the HCRA panel, which was to perform a weight-of-the-evidence evaluation of available data on the developmental and reproductive effects of exposure to BPA in laboratory animals, led to an analysis of only 19 of 47 available published studies on low-dose effects of BPA. The deliberations of the HCRA were in 2001–2002, and accordingly, a cut-off date of April 2002 was selected for consideration of the published literature. It is regrettable that the relevance of the analysis was further undermined by a delay of 2.5 years in publication of the report. During the intervening time, between April 2002 and the end of 2004, a large number of additional articles reporting low-dose effects of BPA in experimental animals have been published. The result is that by the end of 2004, a PubMed (National Library of Medicine, Bethesda, MD) search identified 115 published studies concerning effects of low doses of BPA in experimental animals.

The last U.S. Environmental Protection Agency (EPA) risk assessment for BPA was based on research conducted in the 1980s [Integrated Risk Information System (IRIS) 1988]. The most recent risk assessment of BPA was based on a comprehensive review of the scientific literature conducted in 1998 by the European Union, with some selected articles added through 2001, at which time few of the 115 low-dose BPA studies had been published [European Chemicals Bureau (ECB) 2003]. Below, we describe recent findings concerning mechanisms mediating effects of very low doses of BPA, the adverse effects being reported in animals, and recent findings from human studies. These published findings lead us to strongly recommend that a new risk assessment for BPA be initiated.

The Definition of "Low Dose"

The U.S. EPA considers "low-dose" effects of environmental endocrine-disrupting chemicals to refer to effects being reported for chemicals at doses lower than those used in traditional toxicologic studies conducted for risk assessment purposes. For BPA, the lowest dose studied for risk assessment purposes was 50 mg/kg/day, which is the currently accepted lowest observed adverse effect level (LOAEL) that was used to calculate a reference dose of 50 μg/kg/day based on experiments conducted in the 1980s (IRIS 1988).

BPA is often described as a very "weak" estrogen because in a few assay systems, such as MCF-7 breast cancer cells in culture, the dose of BPA required to stimulate cell proliferation (~ 10^{-7} M or 23 ppb) is roughly 100,000 times higher relative to estradiol, which stimulates cell proliferation at approximately 10^{-12} M (Welshons et al. 1999). This contrasts, however, with the stimulation by BPA of calcium influx in MCF-7 cells that was significant at the lowest dose tested, which was 10^{-10} M or 23 ppt (Walsh et al. 2005). BPA also stimulated calcium influx and prolactin secretion in rat pituitary tumor cells at the lowest dose tested (10^{-12} M or 0.23 ppt), and the magnitude of the response to BPA was similar to the response to the same dose of estradiol (Wozniak et al. 2005). It is difficult to conceive how a chemical that can alter cell function at concentrations < 1 ppt can be characterized as a "weak" endocrine disruptor.

Low-dose effects of endocrine-disrupting chemicals such as BPA are mediated by endocrine-signaling pathways that evolved to act as powerful amplifiers, with the result that large changes in cell function can occur in response to extremely low concentrations (Welshons et al. 2003). Thus, information concerning the *in vivo* potency of estradiol is critical with regard to predicting the *in vivo* bioactivity of chemicals such as BPA. *In vivo* potency of estrogenic chemicals is determined by the affinity of the chemical for the specific type of estrogen receptor (ER) that mediates the effect, the rate of absorption and metabolism, and binding of the chemical to plasma estrogen-binding proteins. The initial interest in low-dose effects of BPA was based on the observation that BPA showed limited binding to plasma estrogen-binding proteins (Nagel et al. 1997), which results in higher free plasma BPA relative to estradiol. It is well known that it is the free hormone level in blood that is predictive of biologic activity (Nagel et al. 1999). A much higher free BPA concentration in blood relative to estradiol would not be taken into account in predicting its *in vivo* potency based simply on cell culture studies conducted in culture medium.

Before conducting the first low-dose *in vivo* study with BPA, vom Saal et al. (1997) found that an increase in size of the fetal mouse prostate occurred in response to an experimental increase in free serum estradiol in fetuses of 0.1 pg/mL serum (0.1 ppt or 0.4×10^{-12} M), from 0.2 pg/mL in control fetuses to 0.3 pg/mL free serum estradiol in estrogen-exposed fetuses. Although this finding was initially controversial, other *in vivo* and *in vitro* studies have since confirmed that very low doses of the estrogenic drug diethylstilbestrol (DES) stimulate an increase in size of the fetal mouse prostate (Gupta 2000; Timms et al. 2005). Nagel et al. (1997) predicted the dose of BPA (fed to pregnant mice) that should be biologically active in mouse fetuses based on a comparison of BPA and estradiol in terms of both the relative affinity for nuclear ERs and binding to serum estrogen-binding proteins that effectively restrict estradiol (but not BPA) uptake into cells. This has been referred to as a "physiologic approach" to dose selection (vom Saal et al. 1998). Nagel et al. (1997) chose the fetal prostate growth bioassay to test the physiologically based prediction of low-dose estrogenic activity of BPA, although the prediction was that any estrogenic response would be altered by exposure to BPA during early development. Nagel et al. (1997) reported finding an enlarged prostate in male offspring after feeding pregnant mice 2 or 20 μg/kg/day BPA. Because these doses are below the current reference dose, this finding received a considerable amount of attention.

The findings by Nagel et al. (1997) raised a critical question: Why were the estrogenic effects that they observed below the current reference dose not predicted based on traditional toxicologic studies that focused on the toxic effects of very high doses of BPA (Morrissey et al. 1987)? The toxicologic approach involves dose selection based on the maximum tolerated dose, which can be described as "top-down dose selection," whereas the physiologic approach used by Nagel et al. (1997) can be described as "bottom-up dose selection" (Welshons et al. 2003). We show below that there is now overwhelming evidence demonstrating that these different experimental approaches lead to very different conclusions of safety with regard to the current reference dose for BPA of 50 μg/kg/day. Findings based on low-dose studies thus present a strong challenge to the assumptions that form the basis for chemical risk assessments.

Why Did the APC Contract with the HCRA to Write a Report on Low-Dose Effects of BPA?

The controversy created by reports of findings for BPA and other chemicals at "low doses," and studies funded by chemical corporations that quickly disputed these findings, resulted in the U.S. EPA asking the National Toxicology Program (NTP) to host a meeting in October 2000 on the low-dose issue. The final NTP Low Dose Peer Review report (NTP 2001) was summarized by the co-chairs and session organizers (Melnick et al. 2002).

In contrast to today, at the time of the NTP low-dose meeting there were relatively few published low-dose studies with BPA. However, the NTP report (NTP 2001) was critical of some of the industry-funded studies of BPA. For example, one industry-sponsored study (Ashby et al. 1999) was criticized by the NTP panel (NTP 2001, p. A9) for not identifying that the body weights and reproductive organ weights of the control animals were significantly different from those of control

animals used in the study that it was supposed to replicate (Nagel et al. 1997), and that the study was not a true replication because of the use of different animal feed. Another industry-funded study that concluded that all findings were not statistically significant (Elswick et al. 2000) was harshly criticized by the NTP panel as presenting conclusions that were "flawed," "illogical," and "misleading," and the NTP panel concluded that results were, in fact, statistically significant (NTP 2001, p. A89).

When the initial report of the NTP panel was released, the APC quickly issued a public letter in which the conclusion of the NTP panel—that there was "credible evidence of low-dose effects"—was described as "troubling . . . if not erroneous" (Bisphenol A Global Industry Group 2000). The APC then contracted with the HCRA in 2000, which established a panel of scientists (including coauthor C.H.) to perform a weight-of-the-evidence evaluation of available data on the developmental and reproductive effects of exposure to BPA in laboratory animals. In turn, the HCRA panel focused on 19 published studies of the available 47 publications and, particularly, on the effects of low doses of BPA on development of the reproductive system in male rodents. The conclusions in the panel's published report (Gray et al. 2004) were directed to this portion of the literature that was intensively scrutinized, but the wording was promptly interpreted by plastic industry trade organizations as suggesting that a far more complete survey of the BPA literature had been encompassed by the panel's review process (APM 2005; vom Saal 2005). As of April 2002, there were 47 available publications that could have been examined in a comprehensive review of all low-dose effects of BPA in all species. Because of the charge to the HCRA panel and its response to that charge, it reviewed 7 of 9 (78%) of the industry-funded published studies, but reviewed only 12 of 38 (38%) of the government-funded studies that were available in the published literature.

Current Status of Literature on Low-Dose Effects of BPA

Of a total of 115 published studies with low doses of BPA below the prior LOAEL of 50 mg/kg/day that we accessed via a PubMed search at the end of December 2004, there have been 94 published studies reporting *in vivo* estrogenic activity of BPA. Of the 94 low-dose studies reporting significant effects, 31 published studies have reported effects caused by doses of BPA at and below the reference dose of 50 µg/kg/day.

Rate of growth and sexual maturation, hormone levels in blood, reproductive organ function, fertility, immune function, enzyme activity, brain structure, brain chemistry, and behavior are all affected by exposure to low doses of BPA. Many of these effects are due to exposure during early development (gestation and/or lactation), but effects due to postweaning-through-adult exposure have also been reported.

A comprehensive review of the rapidly growing literature on adverse health effects of low doses of BPA in vertebrates and invertebrates is beyond the scope of this commentary, but references are available online (Endocrine Disruptors Group 2005). We describe below some examples of effects of low doses of BPA in mice and rats.

• Increased postnatal growth in both males and females occurred at maternal doses between 2.4 and 500 µg/kg/day (Honma et al. 2002; Howdeshell et al. 1999; Nikaido et al. 2004; Takai et al. 2000).
• Early onset of sexual maturation in females occurred at maternal doses between 2.4 and 500 µg/kg/day (Honma et al. 2002; Howdeshell et al. 1999; Nikaido et al. 2004).
• Altered plasma luteinizing hormone levels occurred at a maternal dose of 2 µg/kg/day (Akingbemi et al. 2004), and decreased plasma testosterone in males occurred at a maternal dose of 2 µg/kg/day (Akingbemi et al. 2004; Kawai et al. 2003).
• An increase in prostate size in male offspring occurred at maternal doses between 2 and 50 µg/kg/day (Gupta 2000; Nagel et al. 1997; Timms et al. 2005). A decrease in daily sperm production and fertility in males was also reported at doses between 0.2 and 20 µg/kg/day due to developmental or adult exposure (Al-Hiyasat et al. 2002; Chitra et al. 2003; Sakaue et al. 2001; vom Saal et al. 1998).
• Stimulation of mammary gland development in female offspring occurred at the very low maternal dose of 0.025 µg/kg/day delivered tonically by an Alzet pump (Markey et al. 2001a). Significant disruption of the alignment of chromosomes during meiosis was observed in developing oocytes during puberty because of leaching of BPA from polycarbonate drinking bottles at doses between 15 and 70 µg/kg/day (Hunt et al. 2003), and an increase in mortality of embryos occurred at a maternal dose of 25 µg/kg/day (Al-Hiyasat et al. 2004). Disruption of adult estrous cycles occurred at maternal doses between 100 and 500 µg/kg/day (Nikaido et al. 2004; Talsness et al. 2000).
• Altered immune function occurred at doses between 2.5 and 30 µg/kg/day (Sawai et al. 2003; Yoshino et al. 2003, 2004).
• A decrease in antioxidant enzymes occurred at the very low dose of 0.2 µg/kg/day in adult males (Chitra et al. 2003).
• Changes in the brain include an increase in progesterone receptor mRNA levels at 400 µg/kg/day (Funabashi et al. 2003), ER-α levels at 40 µg/kg/day (Aloisi et al. 2001), and ER-β mRNA levels at 25 µg/kg/day (Ramos et al. 2003) and a change in brain somatostatin receptors at 400 µg/kg/day (Facciolo et al. 2002).
• Behavioral effects include hyperactivity at 30 µg/kg/day (Ishido et al. 2004), an increase in aggressiveness at 2–40 µg/kg/day (Farabollini et al. 2002; Kawai et al. 2003), altered reactivity to painful or fear-provoking stimuli at 40 µg/kg/day (Aloisi et al. 2002), and impaired learning at 100 µg/kg/day (Negishi et al. 2004). Developmental exposure to BPA also resulted in a significant change in the locus coeruleus, where BPA at 30 µg/kg/day reversed the normal sex differences in this brain structure and eliminated sex differences in behavior (Kubo et al. 2003). Developmental exposure decreased maternal behavior at 10 µg/kg/day (Palanza et al. 2002), altered play and other socio-sexual behaviors at 40 µg/kg/day (Aloisi et al. 2002; Dessi-Fulgheri et al. 2002), and enhanced the behavioral response to drugs such as amphetamine at 40–300 µg/kg/day (Adriani et al. 2003; Suzuki et al. 2003).

Factors Accounting for the Absence of Significant Effects in Low-Dose BPA Experiments

As of the end of 2004, we are aware of 21 studies that report no harm in response to low doses of BPA. Source of funding is highly correlated with positive or negative findings in published articles. For government-funded published studies, 94 of 104 (90%) report significant effects at doses of BPA < 50 mg/kg/day. No industry-funded studies (0 of 11, or 0%) report significant effects at these same doses (Table 1). It is thus reasonable to pose two questions: *a*) Are government-funded scientists under real or perceived pressure to find or publish only data suggesting adverse outcomes? *b*) Are industry-funded scientists under real or perceived pressure to find or publish only data suggesting negative outcomes?

It is important to determine what specific factors, other than just source of funding, are associated with reports of no significant effects of low doses of BPA. In this article we discuss four issues, some of which have became apparent because of findings published after the cut-off date for the literature review in the HCRA report: strain of experimental animal, misinterpretation of finding no significant effects for the positive controls, animal feed, and specific end point examined.

Strain of experimental animal. The importance of the strain of animal used in low-dose BPA research was acknowledged in the HCRA report (Gray et al. 2004) as well as the previous NTP report (NTP 2001). The NTP panel emphasized the need to test for the sensitivity

of any animal model by including a positive control, such as the well-characterized estrogenic drugs DES and ethinylestradiol, and stated that

> Because of clear species and strain differences in sensitivity, animal model selection should be based on responsiveness to endocrine active agents of concern (i.e., responsive to positive controls), not on convenience and familiarity. (NTP 2001, p. vii)

A recent study has revealed the very low sensitivity to any estrogen of the Charles-River Sprague-Dawley (CD-SD) rat used in two studies (Ema et al. 2001; Tyl et al. 2002) that were heavily relied on by the HCRA panel in drawing the conclusion "that the negative findings for reproductive endpoints for rats are more compelling than the positive findings" (Gray et al. 2004). According to Charles River Laboratories (2004), rats were purchased by Charles River from Sprague-Dawley in 1950. This colony was continuously subjected to selective breeding for rapid postnatal growth and large litter size, and then in both 1991 and 1997 new colonies were established from selected animals.

Yamasaki et al. (2002) reported that the CD-SD strain of rat showed some responses to 50-μg/kg/day ethinylestradiol administered for 28 days, and more responses to the very high dose of 200 μg/kg/day. Ethinylestradiol is the potent estrogenic drug used by women in birth control pills at a dose of 0.5 μg/kg/day (based on a body weight of 60 kg). The CD-SD rat thus has a very low sensitivity to ethinylestradiol, because relative to women, it requires 100- to 400-fold higher doses to produce effects. In contrast, the fetal male CF-1 mouse examined in the initial vom Saal laboratory studies with BPA responded to ethinylestradiol with significant changes in adult sperm production and prostate size at a maternal oral dose of 0.002 μg/kg/day (Thayer et al. 2001). The CF-1 male mouse fetus is thus between 25,000 and 100,000 times more sensitive to ethinylestradiol relative to the CD-SD rat. Yamasaki et al. (2002) also reported that 600 mg/kg/day BPA was required to see effects in CD-SD rats. This dose is > 200,000 times higher than the BPA doses used in studies conducted in the vom Saal laboratory (Howdeshell et al. 1999, Nagel et al. 1997), and as indicated above, it is also dramatically higher than doses of BPA required to cause effects in > 90 other low-dose BPA studies conducted with other types of rats, various

mouse strains, and other experimental vertebrate and invertebrate animals. There are now many studies that have been conducted with rats other than the CD-SD strain that show low-dose effects of BPA, but very few of these studies were subject to review by the HCRA panel (Gray et al. 2004).

All studies with CD-SD rats report the absence of significant effects of low doses of BPA (Table 1), although the conclusions in one these studies (Elswick et al. 2000) were questioned by the NTP panel (NTP 2001). If the studies that used the CD-SD rat are eliminated from consideration, 94 of 98 (96%) government-funded studies report significant effects of low doses of BPA, whereas 0 of 8 (0%) industry-funded studies reports significant effects with the same low doses (Table 1).

Misinterpretation of the absence of significant findings for the positive controls. The very low sensitivity of the CD-SD rat strain to BPA was predicted by its low sensitivity to ethinylestradiol when it was included as a positive control. Two industry-funded studies (Ashby et al. 1999; Cagen et al. 1999) were designed with DES included as a positive control, which was reported by industry spokesmen (Toloken 1998) at a public news briefing about the Cagen et al. (1999) study. A critique [Environmental Data Services (ENDS) 1998] pointed out that the positive control, DES, failed to show a difference from the negative controls in each of these studies (Ashby et al. 1999; Cagen et al. 1999); however, the authors did not indicate in their published articles that DES had been used as the positive control. Subsequent studies funded by chemical corporations, all of which have reported the absence of significant effects for low doses of BPA, avoided this problem by simply not including a positive control in the experiment.

The NTP panel (NTP 2001) commented on the issue regarding

> . . . a study in which the positive control does not produce the expected positive response. The prudent course of action in such cases may be to declare the study inadequate and repeat it, regardless of the experimental outcome in the test groups. (NTP 2001, pp. 5–10)

The NTP panel went on to note that,

> For those studies that included DES exposure groups, those that showed an effect with BPA showed a similar low-dose effect with DES (e.g., prostate and uterus enlargement in mice), while those that showed no effect with BPA also found no effect with DES.

As articulated by the NTP panel (NTP 2001), only by including a known estrogenic chemical, such as DES or ethinylestradiol, as a positive control in an experiment can the reason for the failure to find low-dose estrogenic effects of BPA be determined to be due to either inactivity of the chemical, insensitivity of the model animal, or some other variable, such as the type of feed used.

Disruption of low-dose studies of endocrine-disrupting chemicals by variability in components of commercial animal feed. A critical issue in experiments concerning effects of low doses of estrogenic chemicals is that a common rodent feed used in toxicologic studies has been reported by investigators at the National Institute of Environmental Health Sciences (Thigpen et al. 2003) to be highly variable in its estrogenic activity. These investigators reported that some batches of this feed were able to interfere with the ability to detect puberty-accelerating effects of DES in female CD-1 mice, due to the feed maximally advancing the age at puberty in control females (Thigpen et al. 2003). The use of this particular feed by Cagen et al. (1999) and Tyl et al. (2002) raises the possibility that endocrine-disrupting components in this feed played a role in the failure of these studies to show low-dose effects of BPA; Cagen et al. (1999) also failed to find significant effects of the positive control DES, whereas Tyl et al. (2002) did not include a positive control. The HCRA panel (Gray et al. 2004) relied heavily on both of these studies.

Both the NTP (2001) and HCRA panels (Gray et al. 2004) raised the possibility that the type of feed used in some studies may have affected the results, but the information provided by Thigpen et al. (2003) about variability in estrogenic activity in different batches of a feed commonly used in toxicologic studies, and other recent findings regarding variability in endocrine-disrupting components of feed other than phytoestrogens (vom Saal et al. 2004, 2005), was not available for either the NTP or the HCRA panel to review. Thus, it is understandable that the HCRA report could state: "Nor is there compelling evidence that the type of feed administered . . . can explain the negative results reported" (Gray et al. 2004).

It is now clear that it is necessary to develop a standard feed that is appropriate for studies involving the examination of end points that are sensitive to estrogenic chemicals, because estrogenic as well as other components of feed can be present in highly variable amounts in different batches; levels of phytoestrogens in plants vary in response to different environmental conditions. The findings reported by Thigpen et al. (2003) that effects of DES could be masked by some batches of a commercial feed clearly demonstrate that, without an

Table 1. Biased outcome due to source of funding in low-dose *in vivo* BPA research as of December 2004.

Source of funding	All studies		CD-SD rat studies		All studies except CD-SD rats	
	Harm	No harm	Harm	No harm	Harm	No harm
Government	94 (90.4)	10 (9.6)	0 (0%)	6 (100)	94 (96)	4 (4)
Chemical corporations	0 (0)	11 (100)	0 (0%)	3 (100)	0 (0)	8 (100)

Values shown are no. (%).

appropriate positive control, false-negative findings can occur that lead to the false conclusion that even biologically active doses of potent estrogenic drugs such as DES have no effect.

The uterotrophic response is not stimulated by low doses of BPA. Seven articles have reported that low doses of BPA do not stimulate a uterotrophic response (Ashby and Odum 2004; Diel et al. 2004; Gould et al. 1998; Laws et al. 2000; Markey et al. 2001b; Mehmood et al. 2000; Tinwell et al. 2000). For example, a dose of 100 mg/kg/day BPA injected subcutaneously was required to stimulate an increase in uterine weight in prepubertal CD-1 mice (Markey et al. 2001b). This is in marked contrast to the fetal CD-1 mouse prostate (Gupta 2000; Timms et al. 2005), testes (Kawai et al. 2003), mammary glands (Markey et al. 2001a), and brain (Palanza et al. 2002), which all respond to doses of BPA at and far below the reference dose of 50 µg/kg/day. In order to assess the effects of low doses of BPA or other estrogenic endocrine-disrupting chemicals on the uterus, more sophisticated approaches are required than just measuring uterine weight (Markey et al. 2001b; Newbold et al. 2004).

Implications for Risk Assessments of Low-Dose BPA Effects

As noted above, BPA is a widely used chemical, with a capacity in excess of 6.4 billion lb in 2003. If regulatory agencies were to determine that the actual LOAEL for BPA is below the current reference dose of 50 µg/kg/day, the 15 corporations that manufacture BPA would be affected economically (Burridge 2003). However, corporations that manufacture products made from BPA would be less affected because alternatives to BPA already exist for many products. Potential economic impacts need to be considered in relation to the implications for human health of the wide range of adverse effects caused by exposure to very low doses of BPA in the animal experiments described above.

Measurements of current human contamination indicate that exposure of human fetuses to BPA already occurs at levels within the range demonstrated to cause adverse effects in fetal rodents (Schonfelder et al. 2002). Specifically, Zalko et al. (2002) injected pregnant CD-1 mice subcutaneously on gestation day 17 with 25 µg/kg tritiated BPA; parent (unconjugated) BPA levels in mouse fetuses at 0.5, 2, and 24 hr after administration were 4.20, 0.48, and 0.13 ng/g (ppb), respectively. Schonfelder et al. (2002) reported that parent BPA levels in human fetal serum ranged from 0.2 to 9.2 ng/mL (ppb), and the median was 2.3 ng/mL (ppb). Many adverse effects have been reported in offspring due to maternal doses of ≤ 25 µg/kg/day in mice.

That the adverse effects being observed at low doses of BPA in animal experiments should be of concern with regard to human health is shown by a study comparing BPA levels in nonobese and obese women in Japan who had normal ovarian function or polycystic ovarian disease. In this case–control study Takeuchi et al. (2004) reported significantly higher blood levels of BPA both in obese women and in women with polycystic ovarian disease. These findings suggest that the adverse effects due to exposure to low doses of BPA in experimental animals may be predictive of adverse effects in adult humans.

The implications of these results extend beyond BPA, because they may lead to requirements that hazard assessments be designed to detect analogous low-dose impacts of other chemicals. Acknowledgment of the existence of the large number of studies showing unique low-dose effects of BPA could lead to the demand that, in designing studies to assess the hazards of all chemicals for risk assessment purposes, a wider range of doses must be examined, as opposed to only a few very high doses based on the maximum tolerated dose. This would require accepting that extrapolation from data on effects at very high doses (based on the linear-threshold model) is not valid for endocrine-disrupting chemicals (vom Saal and Sheehan 1998; Welshons et al. 2003).

We posed above the question concerning why a large number of estrogenic effects have been observed in studies that examined low doses of BPA, but these effects were not predicted based on traditional toxicologic studies that focused on the toxic effects of very high doses of BPA. When evidence of a non-monotonic, inverted-U dose–response relationship is found in a toxicologic study, the results are often identified as not showing a dose–response relationship. Although findings in toxicologic studies that occur within a low-dose range but not at higher doses are typically discounted or, at best, considered to be rare, just for BPA there are currently 11 *in vivo* and *in vitro* examples of unique effects seen at low doses but not at higher doses (e.g., Endocrine Disruptors Group 2005; Oehlmann et al. 2000; Wetherill et al. 2002; Welshons et al. 2003; Wozniak et al. 2005).

The inverted-U dose–response phenomenon shows that dose selection is critical in studies of chemicals such as BPA, and older toxicologic studies that just examined a few very high doses are not relevant for assessing the possibility of unique effects that only occur within a specific low-dose range. The mechanisms mediating qualitative changes in response over a wide range of doses are now being elucidated at multiple levels, such as gene-response profile (Coser et al. 2003), changes in tissue expression of receptors (Gupta 2000), and changes in neuroendocrine

feedback systems (Rubin et al. 2001; Talsness et al. 2000). Low doses of a hormone can stimulate a response, whereas much-higher doses inhibit the same response, and this phenomenon is so well established that it is used in clinical endocrinology to treat diseases (Kappy et al. 1989; Welshons et al. 2003). Although endocrinologists find it plausible that there are unique effects caused by low doses of a chemical with hormonal activity that might not be observed at much higher doses, this has not been recognized by regulatory agencies involved in risk assessments.

It is important to keep in mind that traditional toxicologic testing of chemicals for regulatory purposes requires examination of only a few very high doses of a chemical, which often do not exceed 50-fold below the maximum tolerated dose (vom Saal and Sheehan 1998). The maximum tolerated dose of BPA is very high (~ 1,250,000 µg/kg/day; Morrissey et al. 1987; IRIS 1988). In contrast, a wide range of adverse effects in 31 published studies have been reported in offspring due to administering pregnant mice and rats doses of BPA 25,000 times lower than the maximum tolerated dose (Endocrine Disruptors Group 2005). The conclusion from these published findings is that examining only a 50-fold dose range on the basis of the maximum tolerated dose is a seriously flawed approach for assessing adverse effects of chemicals that are mediated by highly sensitive endocrine-response mechanisms.

Regulatory agencies readily accept that the predicted reference dose is actually "safe" without ever requiring that this dose be verified in an experiment to cause no adverse effects (vom Saal and Sheehan 1998; Welshons et al. 2003). Regulatory agencies need to acknowledge that there is now overwhelming evidence for adverse effects of one of the highest-volume chemicals in commerce below the previously predicted "safe" daily dose for humans. This should lead to the requirement that new risk assessments be conducted to reevaluate the safety of other chemicals, in addition to BPA.

Conclusions and Recommendations

In summary, a comprehensive up-to-date analysis by regulatory agencies is needed to evaluate the potential hazards to humans from exposure to BPA at doses below the prior LOAEL of 50 mg/kg/day; low doses of BPA have now been reported to alter brain chemistry and structure, behavior, the immune system, enzyme activity, the male reproductive system, and the female reproductive system in a variety of animals, including snails, fish, frogs, and mammals. There are also a number of *in vitro* studies showing that the particular type of ER (α or β) and the specific coregulators present in cells can markedly influence the dose of BPA required to stimulate a response

(e.g., Routledge et al. 2000). This is consistent with estrogen-responsive tissues within the same animal showing marked differences in the dose of BPA required to elicit a response.

Not all effects of BPA are mediated by the classical nuclear ERs (α and β). Very low part-per-trillion doses of BPA can stimulate responses in cultured mouse pancreas cells, rat pituitary tumor cells, and human breast cancer cells via rapid induction of calcium uptake (Quesada et al. 2002; Walsh et al. 2005; Wozniak et al. 2005); these same low doses of BPA stimulate proliferation in mouse (Gupta 2000) and human (Wetherill et al. 2002) prostate cells in culture. Nongenomic cell signaling systems involve serial activation of kinases via ligand binding to receptors associated with the cell membrane, and these pathways are known to have tremendous amplifying capacity.

In the now outdated perspective of the HCRA report (Gray et al. 2004), it was stated that "In the case of BPA the only proposed mechanism for low-dose effects is through modulation of the [nuclear] estrogen receptor." Instead, the recent findings concerning the multiple mechanisms of action of BPA show that at concentrations < 1 ppt, BPA activates receptors associated with the plasma membrane of selected target cells. As the BPA "dose at target" increases, various responses in the same or different cells are activated or inhibited (MacLusky et al. 2005), with the specific dose required being dependent on the subtype of nuclear ER and specific coactivators or coinhibitors that are present. At even higher concentrations (parts per billion to parts per million), inhibition of androgen-stimulated and thyroid-hormone–stimulated responses can also occur. That the integrated output across a 1-million-fold dose range can be nonmonotonic (inverted-U shape) is thus not unexpected by scientists who study hormones and hormonally active drugs or chemicals (Welshons et al. 2003). Regulatory agencies that conduct risk assessments have not addressed the implications of nonmonotonic dose–response curves for endocrine-disrupting chemicals with regard to the linear-threshold model currently used to predict "safe" doses for humans.

The *in vitro* findings at low (and even sub) part-per-trillion doses of BPA have to be viewed in relation to potential effects of free (unconjugated and unbound) BPA levels in human blood. Data from numerous studies show that unconjugated BPA in human blood and tissues is in the low part-per-billion range (Endocrine Disruptors Group 2005) and that BPA shows limited binding to human plasma binding proteins that regulate the uptake of estrogenic chemicals into tissues (Nagel et al. 1999). Importantly, new analytical methods have been developed since the

published literature was reviewed in 1998 for the last BPA risk assessment conducted by the European Union (ECB 2003). These new methods have now made it possible to detect BPA in blood within the range that it shows biologic activity, which was not previously the case. There is thus convincing evidence that biologically active levels of BPA in human blood are above the range that has been demonstrated to cause changes in function in human tissues based on *in vitro* studies.

The literature we reviewed shows that the rate of leaching from commonly used BPA-containing products (the lining of tin cans and polycarbonate food and beverage containers) is high enough to result in adverse effects in laboratory animals (Raloff 1999). These recently published findings indicate that the accepted migration limit [recently set by the European Union (ECB 2003)] of 30 ppb BPA from polycarbonate or resins into food and beverages is not sufficiently protective of human health. The case–control study reporting that ovarian disease in Japanese women is related to blood levels of BPA provides a first confirmation of this prediction in adult humans (Takeuchi et al. 2004).

Almost one-half of the low-dose BPA studies have been published in just the last 2 years, and there were only five published low-dose BPA studies as of 1998 when the initial comprehensive literature search was conducted for the last risk assessment conducted by the European Union (ECB 2003). A thorough analysis of the entire published low-dose BPA literature associated with a new risk assessment by regulatory agencies that takes into account the issues discussed here and elsewhere is now warranted (vom Saal 2005; vom Saal and Sheehan 1998; vom Saal et al. 2004, 2005, in press; Welshons et al. 2003). It is important that a reexamination of the BPA low-dose literature include a discussion of the requirement for appropriate positive controls, which was identified by the NTP panel as a significant problem in studies claiming to find no low-dose effects.

The initial NTP review concerning BPA as a carcinogen concluded that "there was no convincing evidence that [BPA] was carcinogenic," because the background level of cancer was high in untreated males (NTP 1982). This conclusion has been criticized by a scientist in the NTP: Huff (2002) concluded that, if these findings (NTP 1982) were officially reanalyzed based on the approach to interpreting cancer in animal studies used today, BPA would be interpreted as being associated with an increase in tumors of blood cells, the testes, and the mammary glands.

In summary, a new risk assessment is needed to establish a new LOAEL and a new reference dose for BPA based on the extensive new information from low-dose studies. In

addition, the low-dose literature for BPA and other endocrine-disrupting chemicals shows that regulatory agencies need to begin the process of reevaluating the assumptions that provide the basis for the linear-threshold model used in risk assessments.

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