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Update on Bisphenol A for Use in Food Contact Applications
U.S. Food and Drug Administration
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Overview

Bisphenol A (BPA) is an industrial chemical that has been present in many hard plastic bottles and metal-based food and beverage cans since the 1960s.

Studies employing standardized toxicity tests have thus far supported the safety of current low levels of human exposure to BPA. However, on the basis of results from recent studies using novel approaches to test for subtle effects, both the National Toxicology Program at the National Institutes of Health and FDA have some concern about the potential effects of BPA on the brain, behavior, and prostate gland in fetuses, infants, and young children. In cooperation with the National Toxicology Program, FDA's National Center for Toxicological Research is carrying out in-depth studies to answer key questions and clarify uncertainties about the risks of BPA.

In the interim:

- FDA is taking reasonable steps to reduce human exposure to BPA in the food supply. These steps include:
 - supporting the industry's actions to stop producing BPA-containing baby bottles and infant feeding cups for the U.S. market;
 - facilitating the development of alternatives to BPA for the linings of infant formula cans; and
 - supporting efforts to replace BPA or minimize BPA levels in other food can linings.
- FDA is supporting a shift to a more robust regulatory framework for oversight of BPA.
- FDA is seeking further public comment and external input on the science surrounding BPA.

FDA is also supporting recommendations from the Department of Health and Human Services for infant feeding and food preparation to reduce exposure to BPA.

FDA is not recommending that families change the use of infant formula or foods, as the benefit of a stable source of good nutrition outweighs the potential risk from BPA exposure.

Background

BPA is an industrial chemical used to make a hard, clear plastic known as polycarbonate, which has been used in many consumer products, including reusable water bottles and baby bottles. BPA is also found in epoxy resins, which act as a protective lining on the inside of metal-based food and beverage cans. These uses of BPA are subject to premarket approval by FDA as indirect food additives or food contact substances. The original approvals were issued under FDA's food additive regulations and date from the 1960s.

Studies employing standardized toxicity tests used globally for regulatory decision making thus far have supported the safety of current low levels of human exposure to BPA.¹ However, results of recent studies using novel approaches and different endpoints describe BPA effects in laboratory animals at very low doses corresponding to some estimated human exposures.² Many of these new studies evaluated developmental or behavioral effects that are not typically assessed in standardized tests.

The National Toxicology Program Center for the Evaluation of Risks to Human Reproduction, part of the National Institutes of Health, completed a review of BPA in September 2008.³ The National Toxicology Program uses five different terms to describe its level of concern about the different effects of chemicals: negligible concern, minimal concern, some concern, concern, and serious concern.⁴

In its report on BPA, the National Toxicology Program expressed “*some concern* for effects on the brain, behavior, and prostate gland in fetuses, infants, and children at current human exposures to bisphenol A.”⁵ The Program also expressed “*minimal concern* for effects on the mammary gland and an earlier age for puberty for females in fetuses, infants, and children at current human exposures to bisphenol A” and “*negligible concern*” for other outcomes.⁶

The National Toxicology Program does not make regulatory recommendations. With respect to neurological and developmental outcomes of BPA, the Program stated that “additional research is needed to more fully assess the functional, long-term impacts of exposures to bisphenol A on the developing brain and behavior.”⁷ The Program also stated:

Overall, the current literature cannot yet be fully interpreted for biological or experimental consistency or for relevance to human health. Part of the difficulty for evaluating consistency lies in reconciling findings of different studies that use different

¹ See, e.g., European Food Safety Authority. Toxicokinetics of Bisphenol A, Scientific Opinion of the Panel on Food additives, Flavourings, Processing aids and Materials in Contact with Food, Adopted 9 July 2008, *The EFSA Journal* 2008. Available online at http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/afc_ej759_bpa_%20toxicokinetics_op_en.pdf?ssbinary=true.

² See, e.g. vom Saal FS, Akingbemi BT, Belcher SM et al. Chapel Hill bisphenol A expert panel consensus statement: integration of mechanisms, effects in animals and potential to impact human health at current levels of exposure, *Reproductive Toxicology* 2007;24:131-8.

³ NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A, NIH Publication No. 08-5994, September 2008.

⁴ *Ibid*, page 6.

⁵ *Ibid*.

⁶ *Ibid*.

⁷ *Ibid*, page 20.

experimental designs and different specific behavioral tests to measure the same dimension of behavior.⁸

In August 2008, prior to the release of the final National Toxicology Program report, FDA released a document entitled *Draft Assessment of Bisphenol A for Use in Food Contact Applications*.⁹ This draft assessment was then reviewed by a Subcommittee of FDA's Science Board, which released its report at the end of October 2008.¹⁰

Since that time, the Center for Food Safety and Applied Nutrition within FDA has reviewed additional studies of low-dose toxicity cited by the National Toxicology Program and the Science Board Subcommittee as well as other such studies that have become available. The Center then prepared a document entitled *Bisphenol A (CAS RN. 80-05): Review of Low Dose Studies*, dated August 31, 2009. In the fall of 2009, FDA's Acting Chief Scientist asked five expert scientists from across the federal government to provide independent scientific evaluations of this document.

FDA is continuing to consider the low dose toxicity studies of BPA as well as other recent peer-reviewed studies related to BPA. At this stage, FDA is explaining its current perspective on BPA, its support for further studies, its intent to solicit and consider public comment before revising its assessment of BPA use in food contact applications, its interim public health recommendations, its view of the appropriate regulatory framework for BPA use in food contact applications, and our planned collaborations with international partners.

FDA's Current Perspective on BPA

At this interim stage, FDA shares the perspective of the National Toxicology Program that recent studies provide reason for some concern about the potential effects of BPA on the brain, behavior, and prostate gland of fetuses, infants and children. FDA also recognizes substantial uncertainties with respect to the overall interpretation of these studies and their potential implications for human health effects of BPA exposure. These uncertainties relate to issues such as the routes of exposure employed, the lack of consistency among some of the measured endpoints or results between studies, the relevance of some animal models to human health, differences in the metabolism (and detoxification) of and responses to BPA both at different ages and in different species, and limited or absent dose response information for some studies.

⁸ Ibid.

⁹ U.S. Food and Drug Administration, *Draft Assessment of Bisphenol A for Use in Food Contact Applications*, 14 August 2008 (Available online at http://www.fda.gov/ohrms/dockets/AC/08/briefing/2008-0038b1_01_02_FDA%20BPA%20Draft%20Assessment.pdf).

¹⁰ FDA Science Board Subcommittee on Bisphenol A. *Scientific Peer-Review of the Draft Assessment of Bisphenol A for Use in Food Contact Applications*, 31 October 2008 (Available online at <http://www.fda.gov/ohrms/dockets/ac/08/briefing/2008-4386b1-05.pdf>).

FDA is pursuing additional studies to address the uncertainties in the findings, seeking public input and input from other expert agencies, and supporting a shift to a more robust regulatory framework for oversight of BPA to be able to respond quickly, if necessary, to protect the public.

In addition, FDA is supporting reasonable steps to reduce human exposure to BPA, including actions by industry and recommendations to consumers on food preparation. At this time, FDA is not recommending that families change the use of infant formula or foods, as the benefit of a stable source of good nutrition outweighs the potential risk of BPA exposure.

Additional Studies

FDA supports additional studies, by both governmental and non-governmental entities, to provide additional information and address uncertainties about the safety of BPA.

FDA's Studies. FDA's National Center for Toxicological Research is pursuing a set of studies on the safety of low doses of BPA, including assessment of the novel endpoints where concerns have been raised. These include studies pursued in collaboration with the National Toxicology Program and with support and input from the National Institute for Environmental Health Sciences. Depending on the results, each could influence regulatory decisions about BPA.

FDA's studies include:

- Physiologically based pharmacokinetic modeling studies in both rodents and nonhuman primates are under way to predict internal exposure of BPA in both the free and conjugated forms, and to provide data on the magnitude of inter-individual differences. These data will facilitate comparisons of exposure across all stages of development and development of relationships between the results of rodent and nonhuman primate feeding studies, and will allow comparisons of internal doses of BPA when given by oral and intravenous routes. This approach has been identified as critical in order to fully evaluate the potential human health implications of studies that have used novel endpoints or non-oral dosing, particularly in rodents, which may metabolize BPA differently than humans. These data will also allow the agency to assess the magnitude of the potential differential exposure (and risk) to neonates. Results from this study are expected to be available to FDA to inform the agency's decisionmaking starting in spring 2010.
- Rodent subchronic studies are in progress to characterize potential effects, and, where observed, the dose-response relationship in the prostate and mammary glands for orally administered BPA. In addition, these studies will explore other issues including potential effects of BPA on metabolic changes and cardiovascular endpoints. These studies will include an *in utero* phase, mimic bottle feeding in neonates, and employ a dose range that will cover the low doses where effects have been previously reported in some animal studies, as well as higher doses where estrogenic effects have been measured in guideline oral studies. Results from this study are expected to be available to FDA to inform the agency's decisionmaking starting in 2012.

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- Rodent behavioral/neuroanatomical pilot studies are also already in progress as part of the sub-chronic study to characterize dose levels at which behavioral, neuroanatomical, neurochemical and hormonal endpoints may be affected by developmental exposure to BPA. These data are intended to evaluate possible effects of exposure to BPA during development that have been reported in some published studies on sexually dimorphic behavioral endpoints such as anxiety, as well as on standard developmental neurotoxicity tests. Results from this study are expected to be available to FDA to inform the agency's decisionmaking starting in 2012.

Other Studies. Other studies on the safety of BPA are also underway. For example, the National Institute of Environmental Health Sciences has recently announced that it is providing \$30 million in funding to study BPA, which includes support both for FDA studies and external grants.

Public Comment and Next Steps for FDA's Assessment of BPA

FDA will open a public docket for comment on BPA. The docket will contain the Center for Food Safety and Applied Nutrition's review of the low dose toxicity studies and recently published studies, the five expert reviews, and other relevant material. The agency welcomes comments on these documents, other available evidence, and the agency's regulatory options. This docket will be open for public comment for 60 days.

FDA will also continue to consult with other expert agencies in the federal government, including the National Institutes of Health (and National Toxicology Program), Environmental Protection Agency, Consumer Product Safety Commission, and the Centers for Disease Control and Prevention.

Based on this outside input and the results of new studies, FDA will update its assessment of BPA and will be prepared to take additional action if warranted. As the scientific field is evolving rapidly, FDA anticipates providing further updates on BPA to the public as significant new information becomes available.

Interim Public Health Recommendations

At this interim stage, FDA supports reasonable steps to reduce exposure of infants to BPA in the food supply. In addition, FDA will work with industry to support and evaluate manufacturing practices and alternative substances that could reduce exposure to other populations.

Given that these are preliminary steps being taken as a precaution, it is important that no harmful changes be made in food packaging or consumption, whether by industry or consumers, that could jeopardize either food safety or reduce access to and intake of food needed to provide good nutrition, particularly for infants.

Infants. Infants are a potentially sensitive population for BPA because (1) their neurological and endocrine systems are developing; and (2) their hepatic system for detoxification and elimination of such substances as BPA is immature.

- FDA is supporting the industry's actions to stop producing BPA-containing bottles and infant feeding cups for the U.S. market. FDA understands that over the past year, the major manufacturers of these products have stopped selling new BPA-containing bottles and infant feeding cups for the U.S. market. Glass and polypropylene bottles and plastic disposable "bag" liners have long been alternatives to polycarbonate nursing bottles.
- FDA is facilitating the development of alternatives to BPA for the linings of infant formula cans. FDA has already noted increased interest on the part of infant formula manufacturers to explore alternatives to BPA-containing can linings, and has received notifications for alternative packaging. The agency is supporting efforts to develop and use alternatives by (1) working with manufacturers regarding the regulatory status and safety of alternative liners; (2) giving technical assistance to those wishing to prepare applications for approval of alternatives; and (3) expeditiously reviewing any such new applications for alternatives. Because reliable can lining materials are a critical factor in ensuring the quality of heat processed liquid infant formula, safe replacement of such materials requires not only that they both be safe for food contact but also allow for processing that is fully functional in protecting the safety and quality of the infant formula itself.

The American Academy of Pediatrics and other health authorities recommend breastfeeding as the optimal nutrition for infants. Infant formula, including infant formula packaged in cans, is a safe and acceptable alternative that provides known nutritional benefits and prevents life-threatening nutritional deficiencies.

FDA is not recommending that families change the use of infant formula or foods, as the benefit of a stable source of good nutrition outweighs the potential risk of BPA exposure.

Other Populations. With respect to uses of BPA in packaging of food intended for other populations, FDA will support changes in food can linings and manufacturing to replace BPA or minimize BPA levels where the changes can be accomplished while still protecting food safety and quality. FDA will support efforts to develop alternatives for other can lining applications similar to those which are already being tested for liquid infant formula packaging. Reliable can lining materials are a critical factor in ensuring the quality of heat processed foods. Therefore, FDA will work to encourage and facilitate changes that minimize exposure to BPA and avoid other adverse impacts on food safety or quality.

Other Advice. FDA is supporting recommendations by the Department of Health and Human Services for infant feeding and food preparation to reduce exposure to BPA

The Regulatory Framework for BPA

Current BPA food contact uses were approved under food additive regulations issued more than 40 years ago. This regulatory structure limits the oversight and flexibility of FDA. Once a food additive is approved, any manufacturer of food or food packaging may use the food additive in accordance with the regulation. There is no requirement to notify FDA of that use. For example,

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today there exist hundreds of different formulations for BPA-containing epoxy linings, which have varying characteristics. As currently regulated, manufacturers are not required to disclose to FDA the existence or nature of these formulations. Furthermore, if FDA were to decide to revoke one or more approved uses, FDA would need to undertake what could be a lengthy process of rulemaking to accomplish this goal.

Since 2000, FDA has regulated new food contact substances through the Food Contact Notification Program. Under this program:

- FDA receives notification from each manufacturer of the basis for the safe use of a food contact substance, detailing the conditions of the substance's use, allowing the agency and public to know how much is being used, and for what applications;
- FDA can work with individual manufacturers to minimize exposure if a potential or actual safety concern is identified after approval;
- FDA can require the submission of additional safety and exposure data from individual manufacturers to address a significant safety concern;
- FDA can require additional studies by individual manufacturers to address a significant safety concern; and
- If FDA were to reach a conclusion that revocation of one or more approved uses is justified, FDA could quickly protect the public by revoking the use through a notice published in the Federal Register.

Given concern about BPA, and the ongoing evaluation of and studies on its safety, FDA believes that the more modern framework is more robust and appropriate for oversight of BPA than the current one.

FDA will encourage manufacturers to voluntarily submit a food contact notification for their currently marketed uses of BPA-containing materials.

In addition, FDA will explore additional options to regulate BPA under the more modern framework.

Collaboration with International Partners

FDA will continue to participate in discussions with our International regulatory and public health counterparts who have also been engaged in assessing the safety of BPA.

For example, FDA has participated with Health Canada in encouraging industry efforts to refine their manufacturing methods for the production of infant formula can linings to minimize migration of BPA into the formula.

In addition, FDA is planning to support and participate in an upcoming planned Expert Consultation on BPA to be convened by World Health Organization and the Food and Agriculture Organization of the United Nations. Information about this expert consultation is available from the WHO web site:

http://www.who.int/foodsafety/fs_management/infosan_archives/en/



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Chapel Hill bisphenol A expert panel consensus statement: Integration of mechanisms, effects in animals and potential to impact human health at current levels of exposure

Keywords: Bisphenol A; *In vitro*; *In vivo*; Rat; Mouse; Aquatic animal; Cancer; Low dose; Non-monotonic dose–response curves; Developmental programming

1. Introduction

This document is a summary statement of the outcome from the meeting: “*Bisphenol A: An Examination of the Relevance of Ecological, In vitro and Laboratory Animal Studies for Assessing Risks to Human Health*” sponsored by both the NIEHS and NIDCR at NIH/DHHS, as well as the US-EPA and Commonwealth on the estrogenic environmental chemical bisphenol A (BPA, 2,2-bis(4-hydroxyphenyl)propane; CAS# 80-05-7). The meeting was held in Chapel Hill, NC, 28–30 November 2006 due to concerns about the potential for a relationship between BPA and negative trends in human health that have occurred in recent decades. Examples include increases in abnormal penile/urethra development in males, early sexual maturation in females, an increase in neurobehavioral problems such as attention deficit hyperactivity disorder (ADHD) and autism, an increase in childhood and adult obesity and type 2 diabetes, a regional decrease in sperm count, and an increase in hormonally mediated cancers, such as prostate and breast cancers. Concern has been elevated by published studies reporting a relationship between treatment with “low doses” of BPA and many of these negative health outcomes in experimental studies in laboratory animals as well as *in vitro* studies identifying plausible molecular mechanisms that could mediate such effects. Importantly, much evidence suggests that these adverse effects are occurring in animals within the range of exposure to BPA of the typical human living in a developed country, where virtually everyone has measurable blood, tissue and urine levels of BPA that exceed the levels produced by doses used in the “low dose” animal experiments.

Issues relating to BPA were extensively discussed by five panels of experts prior to and during the meeting, and are summarized in five reports included in this issue: (1) human exposure to bisphenol A (BPA) [1]; (2) *in vitro* molecular mechanisms of bisphenol A action [2]; (3) *in vivo* effects of bisphenol A in laboratory animals [3]; (4) an ecological assessment of bisphenol A: evidence from comparative biology [4]; (5) an evaluation

of evidence for the carcinogenic activity of bisphenol A [5]. Further discussion occurred at the meeting where participants from the panels were reorganized into four breakout groups. The consensus statements from the meeting are presented below.

The definition of “low dose” of BPA at this meeting used the same two criteria established at a prior NIH meeting concerning the low dose endocrine disruptor issue [6]: (1) for laboratory animal studies “low doses” involved administration of doses below those used in traditional toxicological studies conducted for risk assessment purposes. For BPA the lowest dose previously examined for risk assessment purposes was 50 mg (kg⁻¹ day⁻¹) in studies with rats and mice. The 50 mg (kg⁻¹ day⁻¹) dose is the currently accepted lowest adverse effect level (LOAEL) that was used to calculate the current US-EPA reference dose (the daily dose that EPA calculates is safe for humans over the lifetime) of 50 μg (kg⁻¹ day⁻¹). The current reference dose is thus based on “high dose” experiments conducted in the 1980s [7]. (2) “Low dose” also refers to doses within the range of typical human exposure (excluding occupational exposures). For purposes of this meeting, the published literature that was reviewed met both of these criteria for being considered within the “low dose” range.

Hundreds of *in vitro* and *in vivo* studies regarding the mechanisms and effects of low doses of BPA, as well as studies of biomonitoring and sources of exposure, have been published in peer reviewed journals over the last 10 years, since the first “low dose” BPA *in vivo* studies were published [8–10]. The meeting was convened specifically to integrate this relatively new information. This task required the combined expertise of scientists from many different disciplines, and care was taken to ensure that participants covered these diverse areas.

BPA is a high-volume (>6 billion pounds per year) production chemical used to make resins and polycarbonate plastic [11]. Of particular concern is the use of BPA in food and beverage plastic storage and heating containers and to line metal cans. In addition, potential environmental sources of BPA contamination are due to use in dental fillings and sealants [12], losses at the production site [13], leaching from landfill [14,15], and presence in indoors air [16].

BPA has become a chemical of “high concern” only in recent years, even though BPA was shown to stimulate the reproductive

Abbreviations: ADHD, attention deficit hyperactivity disorder; BADGE, bisphenol A diglycidyl ether; BIS-DMA, bisphenol A dimethacrylate; BIS-GMA, bisphenol A glycerolate dimethacrylate; BPA, bisphenol A; ER, estrogen receptor

addition, identify any *in vivo* findings that are unexpected based on the *in vitro* literature.

- Issue (2) Assess the degree to which ecological studies with wildlife are consistent with laboratory studies in similar and different species. For example, determine the similarity of exposure levels and types of responses seen in wildlife and laboratory animals.
- Issue (3) Discuss the degree to which the low doses of BPA used in laboratory animal studies relate to the levels detected in human serum and tissues (including urine).
- Issue (4) Assess the importance of life stage in the pharmacokinetics of BPA, levels of exposure to BPA, and the health effects of BPA in animals and humans.

3. Findings submitted by the four breakout groups

The reports from the breakout groups are presented below. The four breakout groups conducted a critical examination of the published research on BPA in relation to the four topics described above. Each of the breakout groups identified areas of knowledge and research gaps and made suggestions for future directions of research. In addition, each group identified which of the following two categories applied to specific outcomes:

- “We are confident of the following”: this category applied when there were findings reported in multiple papers from multiple labs that were in agreement. There should have been no papers reporting conflicting findings, unless there were flaws in those papers, in which case the flaw(s) should have been identified.
- “We believe the following to be likely but requiring confirmation”: This category applied when there were multiple consistent findings from one lab, or there may have been some conflicting reports along with reports of significant findings.

4. Levels of confidence for published BPA findings

The responses from the four different breakout groups were integrated together and organized based on levels of confidence. The criterion for a statement being included in a category was that there had to be consensus among all four of the breakout groups about the statement.

4.1. Based on existing data we are confident of the following

4.1.1. Issue 1: In vitro mechanistic research—laboratory animal research connection

1. *In vitro* studies have provided two routes of plausibility for low dose *in vivo* effects of BPA. These include binding to nuclear estrogen receptors that regulate transcription as well as estrogen receptors associated with the cell membrane that promote calcium mobilization and intracellular signaling. Receptors associated with the cell membrane are more sensitive to BPA than the nuclear receptors. Actions mediated by membrane associated receptor signaling may underlie much

of the low dose BPA phenomena (effects have been reported at doses as low as 1 pM or 0.23 ppt). This increases the plausibility of effects at low doses, which are within the range of environmentally relevant doses (human and wildlife levels of exposure).

- 2. *In vitro* mechanistic information has informed us that exposing tissues to only an extremely narrow range of doses of BPA may lead to erroneous conclusions. Non-monotonic dose–response curves are encountered frequently in basic endocrinological research, and numerous examples have been reported for BPA reviewed in Refs. [18,23,24]. Because of this animal experiments on unstudied systems must avoid narrow dose ranges, especially the use of only a few very high doses. Thus, testing one or two doses and concluding that there are no effects is inappropriate. At somewhat higher doses than are required for estrogen receptor (ER)-mediated responses, BPA also interacts with androgen and thyroid hormone receptors, making predictions of effects at different doses very complex.
- 3. *In vitro* studies can dissect mechanisms of complicated effects observed *in vivo*. The proposed potential mechanisms acting *in vitro* and *in vivo* are the same, involving estrogen receptor mediated (nuclear- and membrane-associated) actions. However, specific effects are dose and cell/tissue specific. In addition, there are *in vivo* processes that are not reflective of currently known mechanisms that have been identified *in vitro*. This is due to previously unknown mechanisms as well as the complexity (due to interactions among cell and tissue types) of *in vivo* systems.

4.1.2. Issue 2: Wildlife—laboratory animal research connection

- 1. BPA is found in the environment: aquatic, terrestrial and air.
- 2. Studies of wildlife demonstrate estrogenic responses that are similar to responses seen in laboratory animals. Specifically, reductions in spermatogenesis are seen in wildlife at ecological concentrations of BPA, and these effects are also seen in controlled laboratory studies with BPA. In addition, vitellogenin response is a common biomarker in non-mammalian wildlife and laboratory species for BPA-induced estrogen receptor activation as well as activation by other estrogens.
- 3. BPA exposure induces similar effects in reproductive systems in wildlife and experimental animal model systems, but concentrations used in experiments involving wildlife species are often higher than environmental exposures. There are conditions in the environment, such as landfill leachates and effluent outflow that cause episodic exposure of field populations to elevated doses of BPA.
- 4. Responses in a variety of vertebrate wildlife species are qualitatively consistent with controlled laboratory studies with BPA. Thus, animals in the wild show evidence of harm, and controlled laboratory studies with model aquatic animals (i.e., medaka, zebrafish, and fathead minnows) are consistent with observations made in wildlife species. Low dose effects of BPA (low ppb range) have been observed in many of these animals.

4. In wildlife and laboratory studies, BPA induces alteration in steroid biosynthesis/ metabolism/excretion.
5. Wildlife residing in sediment is likely exposed to higher levels of BPA.

4.2.3. Issue 3: Laboratory animal research—human exposure connection

1. Human exposure is likely to be continuous, unlike exposure in most laboratory animal studies of BPA pharmacokinetics.

4.2.4. Issue 4: Life stage—relationship to exposure pharmacokinetics and health effects

1. Clearance of BPA in the fetus is reduced compared to other life stages. Different effects and metabolic clearance mechanisms are also observed in neonatal and adult animals. Conjugation (glucuronidation) and other mechanisms of metabolic clearance of BPA thus vary throughout life.
2. Exposure to BPA during different life stages differentially influences reproductive cancer etiology and progression, and exposure during sensitive periods in organogenesis may increase susceptibility to development of cancers in some organs, such as the prostate and mammary glands.
3. Early life exposure to environmentally relevant BPA doses may result in persistent adverse effects in humans.
4. The function of the immune system can be altered following adult exposure to BPA.
5. Effects on insulin metabolism occur following adult exposure.

4.3. Areas of uncertainty and suggestions for future research

4.3.1. Issue 1: In vitro mechanistic research—laboratory animal research connection

1. Since BPA can act as an agonist or an antagonist in different tissues and against different background physiological states, the specific co-regulators that mediated these different responses of BPA need to be elucidated based on *in vitro* mechanistic studies, which should be confirmed *in vivo*.
2. Research is needed on specific receptor sub-types (i.e., classical nuclear and non-classical membrane-associated estrogen receptors) in relation to the potency of BPA in different tissues.
3. The identification of multiple estrogen receptor genes and variants as well as different co-regulators with different activities reveals that different levels of potency of BPA could be obtained by complex interactions between these different components that would not be predicted in homogeneous recombinant systems.

4.3.2. Issue 2: Wildlife—laboratory animal research connection

1. To directly relate the effects seen in wildlife with BPA exposure, biomonitoring data are needed from wildlife. In addition

to BPA levels, these studies should assay total estrogenic and antiandrogenic activity from other contaminants.

2. There is a need to examine sensitive endpoints in wildlife that have been identified in laboratory animals.
3. There are substantial amounts of plastic debris within marine and fresh water ecosystems, and studies are needed to examine the impact of BPA in the environment on aquatic organisms. Doses used in laboratory experiments involving wildlife should reflect environmental exposures.
4. More studies need to be done with BPA in invertebrates, and a fundamental understanding of estrogen action in invertebrates is required.
5. Studies should determine if amplification of BPA through the food chain occurs, particularly under anaerobic or hypoxic conditions due to the lack of microbial or photodegradation.
6. Future research emphasis should be placed on populations of aquatic animals exposed to landfill leachate and sewage effluent, as these are the primary point sources for BPA exposure.

4.3.3. Issue 3: Laboratory animal research—human exposure connection

1. Even though there have been attempts to estimate daily human intake of BPA, these estimates require many assumptions. The best measures we have to estimate whether humans may be affected by current exposures to BPA are levels in blood (not exposure levels), which can be related to blood levels in experimental animals after acute exposures. Known sources of human exposure to BPA do not appear sufficient to explain levels measured in human tissues and fluids.
2. While BPA is not persistent in the environment or in humans, biomonitoring surveys indicate that exposure is continuous. This is problematic because acute animal exposure studies are used to estimate daily human exposure to BPA, and at this time, we are not aware of any studies that have examined BPA pharmacokinetics in animal models following continuous low level exposures. Measurement of BPA levels in serum and other body fluids suggests that either BPA intake is much higher than accounted for, or that BPA can bioaccumulate in some conditions such as pregnancy, or both. Research using both animal models, as well as epidemiology studies, are needed to address these hypotheses, and this research needs to better mimic the apparent continuous exposure of humans to BPA.
3. More comprehensive exposure and biomonitoring studies are needed, especially in developing countries.
4. In both animal and human studies, internal exposure measures need to be related to health effects. In particular, there is a need for epidemiological studies relating health outcomes to BPA exposure, particularly during sensitive periods in development. These studies should be based on hypotheses from findings in experimental animals. This will require additional development of appropriate biomarkers in animal studies that can be used in epidemiological research.

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4.3.4. Issue 4: Life stage—relationship to exposure pharmacokinetics and health effects

1. While there is a great need to continue studying prenatal and perinatal exposures in laboratory animal studies, many organs and endpoints continue developing at later stages (throughout puberty and adolescence). Additional studies are needed during these later periods of development.
2. Additional research is needed regarding exposure to BPA in adulthood to determine whether post-exposure effects are temporary or are permanent and associated with subsequent age-related diseases.
3. Because aging adults lose repair mechanisms, metabolic enzymes, and imprinted genes, the possibility that adult exposures (long-term, low level) can increase the risk of cancers and other conditions during aging should be addressed with additional human research and the development of appropriate animal models.
4. Epigenetics should be examined as a potential mechanism mediating developmental effects as well as the trans-generational effects of BPA and other contaminants. Potential effects of adult exposures also need to be examined in relation to disruption of epigenetic changes that occur normally during aging.
5. Trans- and multi-generational effects of BPA must be examined in laboratory animals and humans.
6. There is a need for studies that involve collection of human blood and urine from humans at several life stages, with specific emphasis on infants and young children and continued monitoring throughout adulthood. Additionally, there is a need to characterize the basis for the variability in BPA levels in studies examining both human urine and serum.
7. There is a need for research on the genetic basis for differences in susceptibility to BPA and other contaminants.
8. Studies are needed on comparative BPA pharmacokinetics in invertebrates and vertebrates (non-human primates included).
9. There is a need to measure total endocrine disrupter load in humans and wildlife. Therefore, biomarkers of endocrine disrupter exposure are necessary.
10. There is a need for more research directed at examining human exposure, pharmacokinetics and health effects of selected BPA precursors (i.e., BADGE, BISGMA, and BIS-DMA) and metabolites (e.g., halogenated BPAs).
11. There is a need for more studies focused on identification of other (non-estrogen-receptor mediated) mechanisms of action of BPA.
12. Effects of chemicals on the immune system are life stage dependent, and identifying the life stage dependency for BPA effects on the immune system is necessary. In addition, studies examining BPA effects on the immune system in wildlife are necessary.

5. Conclusions

The published scientific literature on human and animal exposure to low doses of BPA in relation to *in vitro* mechanistic

studies reveals that human exposure to BPA is within the range that is predicted to be biologically active in over 95% of people sampled. The wide range of adverse effects of low doses of BPA in laboratory animals exposed both during development and in adulthood is a great cause for concern with regard to the potential for similar adverse effects in humans. Recent trends in human diseases relate to adverse effects observed in experimental animals exposed to low doses of BPA. Specific examples include: the increase in prostate and breast cancer, uro-genital abnormalities in male babies, a decline in semen quality in men, early onset of puberty in girls, metabolic disorders including insulin resistant (type 2) diabetes and obesity, and neurobehavioral problems such as attention deficit hyperactivity disorder (ADHD).

There is extensive evidence that outcomes may not become apparent until long after BPA exposure during development has occurred. The issue of a very long latency for effects *in utero* to be observed is referred to as the developmental origins of adult health and disease (DOHaD) hypothesis. These developmental effects are irreversible and can occur due to low dose exposure during brief sensitive periods in development, even though no BPA may be detected when the damage or disease is expressed. However, this does not diminish our concern for adult exposure, where many adverse outcomes are observed while exposure is occurring. Concern regarding exposure throughout life is based on evidence that there is chronic, low level exposure of virtually everyone in developed countries to BPA. These findings indicate that acute studies in animals, particularly traditional toxicological studies that only involve the use of high doses of BPA, do not reflect the situation in humans.

The fact that very few epidemiological studies have been conducted to address the issue of the potential for BPA to impact human health is a concern, and more research is clearly needed. This also applies to wildlife, both aquatic and terrestrial. The formulation of hypotheses for the epidemiological and ecological studies can be greatly facilitated by the extensive evidence from laboratory animal studies, particularly when common mechanisms that could plausibly mediate the responses are known to be very similar in the laboratory animal models, wildlife and humans.

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5. The similar effects observed in wildlife and laboratory animals exposed to BPA predict that similar effects are also occurring in humans.

4.1.3. Issue 3: Laboratory animal research—human exposure connection

1. Human exposure to BPA is widespread.
2. Human exposure to BPA is variable, and exposure levels cover a broad range [central tendency for unconjugated BPA: $0.3\text{--}4.4\text{ ng ml}^{-1}$ (ppb)] in tissues and fluids in fetuses, children and adults.
3. Because the current published literature states that there is a linear relationship between administered dose and circulating levels of BPA in animal studies, this allows circulating levels at lower administered doses to be predicted in experimental animals based on the results from studies in which higher doses were administered.
4. All of the currently published metabolic studies in rats predict circulating BPA levels after acute low dose oral exposures at blood levels less than or equal to 2 ng ml^{-1} (ppb), which is the approximate median and mean unconjugated circulating BPA level in humans. Therefore, the commonly reported circulating levels in humans exceed the circulating levels extrapolated from acute exposure studies in laboratory animals.
5. BPA levels in the fetal mouse exposed to BPA by maternal delivery of $25\text{ }\mu\text{g kg}^{-1}$, a dose that has produced adverse effects in multiple experiments, are well within the range of unconjugated BPA levels observed in human fetal blood.

4.1.4. Issue 4: Life stage—relationship to exposure pharmacokinetics and health effects

1. Sensitivity to endocrine disruptors, including BPA, varies extensively with life stage, indicating that there are specific windows of increased sensitivity at multiple life stages. Therefore, it is essential to assess the impact of life stage on the response to BPA in studies involving wildlife, laboratory animals, and humans.
2. Developmental windows of susceptibility are comparable in vertebrate wildlife species and laboratory animals.
3. BPA alters “epigenetic programming” of genes in experimental animals and wildlife that results in persistent effects that are expressed later in life [25]. These organizational effects (functional and structural) in response to exposure to low doses of BPA during organogenesis persist into adulthood, long after the period of exposure has ended. Specifically, prenatal and/or neonatal exposure to low doses of BPA results in organizational changes in the prostate, breast, testis, mammary glands, body size, brain structure and chemistry, and behavior of laboratory animals.
4. There are effects due to exposure in adulthood that occurs at low doses of BPA. Substantial neurobehavioral effects and reproductive effects in both males and females have been observed during adult exposures in laboratory animals.
5. Adult exposure studies cannot be presumed to predict the results of exposure during development.

6. Life stage impacts the pharmacokinetics of BPA.

4.2. We believe the following to be likely but require confirmation

4.2.1. Issue 1: In vitro mechanistic research—laboratory animal research connection

1. BPA metabolism occurs in cell culture systems, and although there are differences between cell types, there is less variability than in the entire animal. Metabolism is an important issue for humans and wildlife field populations with large genetic variability. Individual differences in BPA pharmacokinetics allow for underlying variability within a population, and may allow for the identification of sensitive and insensitive sub-populations.
2. The activity of various enzymes involved in drug, chemical, and hormone metabolism, as well as protection against oxidative stress, are programmed by hormone levels during sensitive periods in development. Developmental alterations in hormonal programming (activation or inhibition) may thus affect metabolism of BPA and other hormones and chemicals. Direct interaction of BPA with enzymes in cells has only been reported at higher doses than expected for human exposures.
3. The set of genes regulated by BPA is expected to differ among doses. Therefore, different doses of BPA do not produce different effects only due to a quantitative difference in the expression of the same set of genes.
4. Differential expression of estrogen receptor subtypes (α/β ; variant isoforms), and protein–protein interactions (estrogen receptor homo- and hetero-dimer formation, co-regulators, etc) modulate the cellular response to BPA. Direct actions of BPA on intracellular signal transduction modulate some cellular responses, which are similarly dependent on differential expression and protein–protein interactions.
5. Bioactive doses can be mathematically modeled, but further model refinement and experimental confirmation is required.
6. Other mechanisms (androgen receptors, thyroid hormone receptors) may be relevant for BPA action, but at higher doses than for estrogen responsive mechanisms.

4.2.2. Issue 2: Wildlife—laboratory animal research connection

1. The effects observed in laboratory animals could be present in wildlife, because the low doses being studied in laboratory animals are now relevant to environmental exposure levels of wildlife. The similarities in mechanisms that have been observed between different species suggest that field populations will respond to the same low levels.
2. Measurements of vitellogenin production in fish have established that there are exogenous estrogenic signals in their environment. BPA may be contributing to this phenomenon as it enters natural water systems after leaching from landfills and due to plastic debris in water.
3. Delayed spawning is seen in male and female fish, which may relate to observed changes in estrous cyclicity in mammals in laboratory experiments.

system in female rats and thus to be an “environmental estrogen” in 1936 [17], long before it was used as the monomer to synthesize polycarbonate plastic and resins in the early 1950s. However, more recent evidence has shown that BPA also exhibits other modes of endocrine disruption in addition to binding to estrogen receptors, such as alterations in endogenous hormone synthesis, hormone metabolism and hormone concentrations in blood. BPA also results in changes in tissue enzymes and hormone receptors, and interacts with other hormone-response systems, such as the androgen and thyroid hormone receptor signaling systems. While BPA was initially considered to be a “weak” estrogen based on a lower affinity for estrogen receptor alpha relative to estradiol [18], research shows that BPA is equipotent with estradiol in its ability to activate responses via recently discovered estrogen receptors associated with the cell membrane [19–22]. It is through these receptors that BPA stimulates rapid physiological responses at low picogram per ml (parts per trillion) concentrations.

2. Purpose and organization of the BPA meeting

2.1. Topic-focused expert panels

To address the strength of the evidence regarding the published BPA research, an organizing committee was formed, and five panels of experts from different disciplines were established. Each panel had a chair or co-chairs and included a scientist who agreed to be primarily responsible, along with the chair, for preparing a preliminary draft of the panel’s report. A web site was established on which all of the available electronic files of articles concerning BPA were posted, along with other pertinent information relating to the meeting. Prior to the meeting, the panel members began working on draft reports and communicated via electronic media and telephone conference calls. The resulting preliminary report from each panel was posted on the web site and distributed at the meeting for all participants to read. After the meeting, each panel completed a manuscript that is a part of this meeting report. These five panel reports were peer reviewed using the normal manuscript submission process to *Reproductive Toxicology*. The following specific concerns about BPA led to the five expert panels being established:

- (1) Leaching of BPA occurs from the resin lining of metal cans and from plastic food and beverage containers under conditions of normal use. BPA is also detected in water and air samples.
- (2) Parts per billion (ppb) levels of BPA that are unconjugated (not metabolized and thus biologically active) are detected in human blood and tissues in different countries, and these levels appear to be higher than blood levels that would be present in animals exposed to the US-EPA reference dose.
- (3) BPA causes a wide range of adverse effects at “low doses” that are below the US-EPA reference dose in animals, both terrestrial and aquatic.
- (4) There is evidence from *in vitro* mechanistic studies that indicates the potential for disruption of human and animal cell

function at concentrations of BPA far below unconjugated levels typically found in human blood and tissues.

- (5) There is evidence that at very low doses, BPA may be carcinogenic or increase susceptibility to cancer in animals.

The five panels each addressed a different topic related to their specific area of expertise with BPA and prepared a panel report that included documentation of the relevant published studies:

- Panel (1) Sources and amounts of human exposure to BPA as well as pharmacokinetics.
- Panel (2) *In vitro* studies related to the molecular mechanisms that mediate responses to BPA with an emphasis on studies using low doses.
- Panel (3) *In vivo* studies of BPA at “low doses” in laboratory animals.
- Panel (4) *In vivo* studies of BPA in aquatic wildlife and laboratory animals.
- Panel (5) Relationship of BPA to cancers.

The purpose of the 3-day meeting was to provide an opportunity for members of the different panels to interact with each other to integrate information from different disciplines concerning low dose effects of BPA after each panel of experts had prepared a report in its specific area. The agenda of the meeting was designed to allow the members of the five panels to have time to discuss the information in their panel reports and finalize statements about the strength of the evidence for the literature that the panel had reviewed.

2.2. Integration of information by breakout groups

For the second part of the meeting the focus was on integrating the information from each of the panel reports. This was accomplished by assigning panel members to one of four breakout groups. The four replicate breakout groups were established using the following criteria, such that each breakout group should have

- (1) At least two members from each of the five panels.
- (2) A person from each panel who had published on BPA.
- (3) A person with general knowledge of endocrine disruption research or endocrinology, but who had not necessarily published on BPA.
- (4) A person with experience in the process of reaching consensus.
- (5) A mixture of junior and senior investigators.

The charge to the replicate breakout groups was to individually integrate the information relating to the following four issues:

- Issue (1) Determine the degree to which the findings on BPA mechanisms of action identify mechanisms and bioactive doses that explain results of the studies reported by the panel on *in vivo* laboratory animal studies. Determine the strength of the evidence for plausible mechanisms mediating *in vivo* effects at low doses. In



Center For The Evaluation of Risks To Human Reproduction

NTP-CERHR MONOGRAPH ON THE POTENTIAL HUMAN REPRODUCTIVE AND DEVELOPMENTAL EFFECTS OF BISPHENOL A

ABSTRACT

NTP-CERHR MONOGRAPH ON THE POTENTIAL HUMAN REPRODUCTIVE AND DEVELOPMENTAL EFFECTS OF BISPHENOL A

The National Toxicology Program (NTP) Center for the Evaluation of Risks to Human Reproduction (CERHR) conducted an evaluation of the potential for bisphenol A to cause adverse effects on reproduction and development in humans. The CERHR Expert Panel on Bisphenol A completed its evaluation in August 2007.

CERHR selected bisphenol A for evaluation because of the:

- Widespread human exposure
- Public concern for possible health effects from human exposures
- High production volume
- Evidence of reproductive and developmental toxicity in laboratory animal studies

Bisphenol A (CAS RN: 80-05-7) is a high production volume chemical used primarily in the production of polycarbonate plastics and epoxy resins. Polycarbonate plastics are used in some food and drink containers; the resins are used as lacquers to coat metal products such as food cans, bottle tops, and water supply pipes. To a lesser extent bisphenol A is used in the production of polyester resins, polysulfone resins, polyacrylate resins, and flame retardants. In addition, bisphenol A is used in the processing of polyvinyl chloride plastic and in the recycling of thermal paper. Some polymers used in dental sealants and tooth coatings contain bisphenol A. The primary source of exposure to bisphenol A for most people is assumed to occur through the diet. While air, dust, and water (including skin contact during bathing and swimming) are other possible sources of exposure, bisphenol A in food and beverages accounts for the majority of daily human exposure. The highest estimated daily intakes of bisphenol A in the general population occur in infants and children.

The results of this bisphenol A evaluation are published in an NTP-CERHR Monograph that includes the (1) NTP Brief and (2) Expert Panel Report on the Reproductive and Developmental Toxicity of Bisphenol A. Additional information related to the evaluation process, including the peer review report for the NTP Brief and public comments received on the draft NTP Brief and the final expert panel report, are available on the CERHR website (<http://cerhr.niehs.nih.gov/>). See bisphenol A under "CERHR Chemicals" on the homepage or go directly to <http://cerhr.niehs.nih.gov/chemicals/bisphenol/bisphenol.html>.

The NTP reached the following conclusions on the possible effects of exposure to bisphenol A on human development and reproduction. Note that the possible levels of concern, from lowest to highest, are negligible concern, minimal concern, some concern, concern, and serious concern.

The NTP has *some concern* for effects on the brain, behavior, and prostate gland in fetuses, infants, and children at current human exposures to bisphenol A.

The NTP has *minimal concern* for effects on the mammary gland and an earlier age for puberty for females in fetuses, infants, and children at current human exposures to bisphenol A.

The NTP has *negligible concern* that exposure of pregnant women to bisphenol A will result in fetal or neonatal mortality, birth defects, or reduced birth weight and growth in their offspring.

The NTP has *negligible concern* that exposure to bisphenol A will cause reproductive effects in non-occupationally exposed adults and *minimal concern* for workers exposed to higher levels in occupational settings.

NTP will transmit the NTP-CERHR Monograph on Bisphenol A to federal and state agencies, interested parties, and the public and make it available in electronic PDF format on the CERHR web site (<http://cerhr.niehs.nih.gov>) and in printed text or CD from CERHR:

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Biomonitoring Studies Should Be Used by Regulatory Agencies to Assess Human Exposure Levels and Safety of Bisphenol A

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BACKGROUND: Within the past 3 years, four major evaluations of bisphenol A (BPA) safety have been undertaken. However, these assessments have arrived at quite different conclusions regarding the safety of BPA at current human exposure levels.

OBJECTIVES: We compared the reasons provided by the European Food Safety Authority (EFSA) BPA risk assessment panel for their conclusion that human exposures are negligible with the conclusions reached by the other panels, with all panels having the same body of literature at their disposal.

DISCUSSION: The EFSA panel dismissed ≥ 80 biomonitoring studies that documented significant levels of BPA exposure in humans, including internal exposures to unconjugated BPA, on the basis that they did not match a model of BPA metabolism. Instead, the EFSA panel relied on two toxicokinetic studies—conducted in 15 adults administered BPA—to draw conclusions about exposure levels in the population, including exposures of neonates.

CONCLUSIONS: As with all exposure assessments, models should be developed to explain actual data that are collected. In the case of BPA, samples from a large number of human subjects clearly indicate that humans are internally exposed to unconjugated BPA. The dismissal of these biomonitoring studies simply because their results do not conform to a model violates scientific principles. Expert panels should evaluate all data—including human biomonitoring studies—to make informed risk assessments.

KEY WORDS: bisphenol A, endocrine disruptor, Good Laboratory Practices, human exposure, regulatory policy, risk assessment, toxicokinetic model. *Environ Health Perspect* 118:1051–1054 (2010). doi:10.1289/ehp.0901717 [Online 5 May 2010]

Bisphenol A (BPA), a component of polycarbonate plastics and epoxy resins, is one of the highest-volume chemicals produced worldwide. Many studies suggest that the amount of BPA to which humans are exposed may cause adverse health effects (reviewed by Bondesson et al. 2009).

The field of endocrine disruption, and particularly BPA research, has been influenced by social issues, legislation, and the media. BPA has attracted the attention of regulatory agencies and scientists around the world because of its estrogenic properties (Wetherill et al. 2007). Since 2006, several panels and agencies have examined the BPA literature and have come to quite different conclusions regarding the safety of human exposure levels [reviewed by Gies et al. (2009) and Vandenberg et al. (2009)]. Specifically, exposure of humans to free (unconjugated) BPA has been questioned. These conflicting decisions seem paradoxical because each was generated using approximately the same literature database.

EFSA Risk Assessment: An Example of Use of Limited Data

As stated in our review (Vandenberg et al. 2010), great concern exists about exposure of human fetuses, infants, and neonates to BPA because of the sensitivity of developing

organs and the brain to exogenous hormones (Vandenberg et al. 2009). However, to translate findings from animal studies to health risks in humans, exposure assessments and biomonitoring of BPA in different populations are essential. Thus, in November 2006, the European Food Safety Authority (EFSA) released its opinion on the plausibility of data regarding levels of BPA in human blood and excretion of BPA and BPA metabolites in environmentally exposed humans. The EFSA panel (2006) concluded that

[T]here is very low oral bioavailability of the parent substance, BPA, in humans and other primates. Due to this rapid biotransformation and excretion and plasma protein binding in humans, peak BPA-concentrations after dietary exposures to BPA available for receptor binding are predicted to be very low even in worst case exposure scenarios.

The EFSA panel was asked to reconsider their assessment based on recent studies that suggested the possibility for age-dependent toxicokinetics of BPA. In July 2008, the EFSA released its second opinion in support of their original statement (EFSA 2008):

The Panel therefore considers that its previous risk assessment ... can be considered as conservative for humans. The Panel concluded that the differences in age-dependent toxicokinetics of BPA in animals and humans would have no implication for the EFSA 2006 risk assessment of BPA.

In stark contrast to these statements, we analyzed > 80 biomonitoring studies and came to the conclusion that measurable levels of BPA and BPA conjugates are present in human blood and urine, as well as in other tissues and fluids (Vandenberg et al. 2010). These biomonitoring studies examined thousands of individuals from many developed and some developing countries and collectively indicate that humans are internally exposed to unconjugated BPA (Vandenberg et al. 2007, 2010; Welshons et al. 2006). Biomonitoring studies are crucial for understanding current human exposure levels because, by their very nature, they account for all exposures. This is essential, because all exposure sources for BPA have not yet been identified, and existing data suggest that non-oral exposures may be significant (Gies et al. 2009; Stahlhut et al. 2009).

A comprehensive review of the large number of biomonitoring studies indicates that they are highly consistent and therefore reliable (Vandenberg et al. 2010). The detection rates and concentrations of BPA in urine and blood of environmentally exposed individuals are remarkably similar in studies performed in many laboratories using a variety of techniques, including highly accurate and sensitive methods [e.g., solid-phase extraction coupled with isotope dilution-HPLC-tandem mass spectrometry, as used by the U.S. Centers for Disease Control and Prevention (Calafat et al. 2005, 2008)]. Further, there is no evidence to suggest that these studies should be invalidated because of poor quality control (e.g., contamination from collection materials, breakdown of conjugates during storage, inadequate

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blanks) (Gies et al. 2009; Vandenberg et al. 2010). In total, the reproducibility of these results indicates that humans are internally exposed to doses of unconjugated BPA, with a central measure of the distribution in the 0.5–3 ng/mL range. In spite of these consistent findings, the EFSA panel came to a completely different conclusion about current human exposures (EFSA 2006, 2008). What is the basis for this disparity?

The pivotal factor we identified in the EFSA report was the bias in the selection of studies used in this evaluation. The EFSA panel (EFSA 2006) ignored the majority of the biomonitoring studies. Although they reviewed 2 toxicokinetic studies (Volkel et al. 2002, 2005) extensively, only 2 of the 17 urine biomonitoring studies published by 2006 were discussed in any detail. Only a small number of the blood biomonitoring studies were cited in the EFSA report, and none of these studies were discussed in detail at any level. Instead, the EFSA panel identified potential problems with these biomonitoring studies, including the use of ELISA (used only in a few studies), possible contamination of reagents with BPA, and the leaching of BPA from materials used for sample collection, storage, and processing. Without providing any evidence that these are indeed issues in the biomonitoring studies examined, the EFSA (2006) concluded that

Due to all these confounders, the reported analytical results on BPA blood concentrations most probably considerably overestimate real blood concentrations actually present.

Of particular concern relative to this stance is that although the biomonitoring studies have produced reliable, consistent results, the two toxicokinetic studies (Volkel et al. 2002, 2005) the EFSA (2006) relied upon heavily for their risk assessment have significant inconsistencies and are yet to be replicated. Most concerning is the fact that the methods used in these two toxicokinetic studies were much less sensitive than those used in almost all biomonitoring studies. The toxicokinetic studies had limits of detection (LODs) as high as 2.28 ng/mL for unconjugated BPA and 10.1 ng/mL for conjugated BPA, compared with LODs of 0.0063–0.4 ng/mL in other studies using similar analytical methods (Vandenberg et al. 2010). These two toxicokinetic studies examined only a small number of adult subjects administered BPA (15 adults total) compared with the thousands of individuals (including infants, children, adolescents, and pregnant women) sampled for biomonitoring purposes. Only one of these studies (Volkel et al. 2002) examined concentrations of BPA in both blood and urine samples, whereas the other study (Volkel et al. 2005) reported conjugated BPA concentrations in urine but provided no information about BPA concentrations in the

plasma samples collected by the authors. Yet both studies were used by the EFSA to discount the presence of BPA in plasma and blood samples reported in numerous other studies (EFSA 2006). Additional problems with data analysis and interpretation in the Volkel et al. studies (2002, 2005) are discussed in greater detail in our review (Vandenberg et al. 2010).

The EFSA panel (2006) speculated that the repeated detection of unconjugated BPA in human blood was due to poor sample processing conditions and/or unreliable methods, stating,

The studies reporting detection of BPA in human blood in concentrations higher than 1 [µg] BPA/L have usually determined [unconjugated] BPA, without prior enzymatic cleavage of BPA-glucuronide.... The fate of BPA-glucuronide under the conditions of the diverse sample processing conditions and a possible cross-reactivity of the [ELISA] antibodies with BPA-glucuronide is not reported, leaving the possibility that reported BPA levels actually reflect BPA-glucuronide levels.

Consistent results from a large number of biomonitoring studies cannot be disregarded based only on the speculation that they overestimated unconjugated BPA levels because of hypothetical poor analytical controls. The deficiencies speculated by the EFSA were addressed and invalidated by one or more appropriate controls within each of the individual biomonitoring studies in question; most studies contained numerous controls to counter speculations of contamination or cross-reactivity of ELISA antibodies. For example, blanks reported in these studies would show measurable BPA if cross-contamination occurred at any step in the sample-handling process or analysis—yet they did not, leaving the speculations made by the EFSA without any scientific basis.

The EFSA panel (EFSA 2006) continued to rationalize their dismissal or lack of attention to biomonitoring studies by referencing the results of toxicokinetic studies:

[O]rally administered BPA is rapidly absorbed from the gastrointestinal tract and undergoes intensive first-pass metabolism to BPA-glucuronide in the gut wall and in the liver.... Concentrations of [unconjugated] BPA were below the limit of detection both in urine ... and blood samples....

Further reasoning provided to reject the findings from biomonitoring studies was that the levels measured in environmentally exposed humans are “higher than the peak BPA concentrations determined in blood of monkeys after oral administration of a dose of 100 µg BPA/kg bw [body weight].” The panel concluded that

[T]hese reported concentrations of BPA in blood of unintentionally exposed human subjects of up to 10 [µg] BPA/L are orders of magnitude above the maximal concentrations of BPA predicted in blood by PBPK [physiologically-based pharmacokinetic] models on the basis of human BPA toxicokinetics after oral administration.

In science, if data contradict the hypothesis (i.e., the model), the hypothesis, not the data, must be rejected. It is unexpected, and perhaps unprecedented, for a scientific body to reject studies because their findings did not match a model, rather than to reconsider the model or reassess the findings from the extremely limited toxicokinetic studies that were used to generate the model. This reasoning is simply not founded in logic and is not how science-based regulatory decisions should be made. Considering the size of the biomonitoring literature, the consistency of the results from biomonitoring studies, and the significant problems in the toxicokinetic studies, conclusions drawn primarily from the two toxicokinetic studies (Volkel et al. 2002, 2005) cannot be valid. Therefore the EFSA conclusion that there is negligible internal exposure to unconjugated BPA has no scientific basis.

The EFSA Panel Inappropriately Extrapolates from Adults to Fetuses and Neonates

Considering the reliance of the EFSA panel on two extremely limited toxicokinetic studies to inform their risk assessment, their statement that “the differences in age-dependent toxicokinetics of BPA in animals and humans would have no implication for the EFSA 2006 risk assessment of BPA” (EFSA 2008) is particularly surprising. The July 2008 EFSA report stated,

The Panel considers that there is sufficient capacity in the neonate to conjugate BPA at doses below 1 mg/kg bw (the Panel noted that exposures at the TDI of 0.05 mg/kg bw are 20 fold lower than this). Therefore, the Panel concluded that there is sufficient capacity for biotransformation of BPA to hormonally inactive conjugates in neonatal humans at exposures to BPA that were considered in the EFSA opinion of 2006 and the European Union Risk Assessment Report.

To date, there are no studies to support this statement. To the contrary, there are many studies that contradict it. First, the two toxicokinetic studies relied upon by EFSA (Volkel et al. 2002, 2005) examined a total of 15 adults (mixed groups of males and females) administered BPA. Although the authors of these studies concluded that there are no kinetic differences between volunteers (Volkel et al. 2005), evaluation of the data presented shows variable metabolic responses after BPA administration. Second, data from biomonitoring studies in different groups of adults clearly indicated differences in urinary concentrations of BPA that are influenced by both sex and age (Calafat et al. 2005, 2008; He et al. 2009). Associations between age and BPA concentrations are also evident from studies that examined children and adolescents; younger children typically

have higher concentrations of BPA metabolites in urine compared with older children and adolescents (Becker et al. 2009; Calafat et al. 2008). Infants in a neonatal infant care unit were found to have total urinary BPA concentrations approximately 11 times higher than those observed in adults (Calafat et al. 2009). Third, researchers using two physiologically based toxicokinetic models that simulated the blood concentration time profile in several age groups predicted that newborns have 3–11 times greater blood BPA concentrations than adults (Edgington and Ritter 2009; Mielke and Gundert-Remy 2009). Finally, a recently published study examining rat fetuses provides evidence that BPA-glucuronide passes from the mother through the placenta and is deconjugated to BPA in the fetus, clearly showing that BPA metabolites can be converted to the biologically active form in the fetus (Nishikawa et al. 2010). A study of human placentas also indicates that unconjugated BPA crossing the placental barrier remains largely in its unconjugated form. Less than 4% of BPA detected in the fetal compartment was conjugated (Balakrishnan et al. 2010).

Similarly, there is little evidence in support of complete conjugation of BPA, even in adults. Six of the seven biomonitoring studies testing for unconjugated BPA in urine found measurable concentrations in at least some individuals examined (Calafat et al. 2009; Kim et al. 2003; Quichi and Watanabe 2002; Schoringhumer and Cichna-Markl 2007; Volkel et al. 2008; Ye et al. 2005). The one study that failed to detect unconjugated BPA examined five pooled urine samples (Brock et al. 2001). One of the toxicokinetic studies relied heavily upon by the EFSA (2006, 2008) also detected unconjugated BPA in the urine of two of the six individuals administered BPA (Volkel et al. 2005). The presence of unconjugated BPA in urine suggests that first-pass metabolism of orally administered BPA may be incomplete, that significant levels of BPA enter the body via routes that circumvent first-pass metabolism, or that BPA metabolites are deconjugated in the body. Importantly, unconjugated BPA has also been measured in fetal umbilical cord blood, amniotic fluid, and placental tissue (Vandenberg et al. 2010). Collectively, these findings clearly indicate that the fetus does not have “sufficient capacity for biotransformation of BPA to hormonally inactive conjugates” (EFSA 2008) and that human adults may not either.

Divergent Conclusions from Other Expert Panels

In the past few years, three other major evaluations of the BPA toxicological database have been undertaken. These expert panels came to seemingly disparate conclusions, yet all

four evaluations took place within a short period of time and had access to essentially the same literature. How is it possible for the same studies to be reviewed so differently by regulatory agencies [the EFSA and the U.S. Food and Drug Administration (FDA)], the National Toxicology Program (NTP), and academic scientists?

The answer lies in how the various panels evaluated the scientific literature. In a previous commentary in *Environmental Health Perspectives*, Myers et al. (2009) described the selection process used by each panel in its assessment of the hundreds of animal studies that, to date, overwhelmingly indicate that developmental exposure to BPA causes adverse effects. The EFSA (2006) and FDA (2008) assessments used only data produced using validated protocols [i.e., studies that conformed to Good Laboratory Practices (GLP)] with the ability to establish no observed adverse effect levels. Although the EFSA and FDA stated that they would use all available data to make regulatory decisions, their guidelines restricted their focus to only a few GLP-compliant studies; all other studies (nearly 1,000 for BPA) were not used because they did not meet this criterion.

Similarly, the EFSA panel (EFSA 2006) clearly divided the human exposure database into two groups: dozens of biomonitoring studies (which did not fit their model of BPA metabolism and were largely ignored or rejected) and two toxicokinetic studies (which fit their model and were used in spite of their higher LODs and small number of individuals examined). The studies used by the other three expert panels and the conclusions reached by each of these panels are discussed below.

Chapel Hill Consensus Statement

In the fall of 2007, a group of scientists from universities and government agencies developed a workshop sponsored by the National Institutes of Health to which experts researching BPA and other endocrine-disrupting chemicals were invited. These academic scientists wrote the Chapel Hill Consensus Statement (vom Saal et al. 2007), which stated, in part, that “the commonly reported circulating levels in humans exceed the circulating levels extrapolated from acute exposure studies in laboratory animals.”

In reaching these conclusions, vom Saal et al. (2007) examined the entire body of scientific data (Crain et al. 2007; Keri et al. 2007; Richter et al. 2007; Vandenberg et al. 2007; Wetherill et al. 2007), including > 40 human biomonitoring studies available at the time and the two human toxicokinetic studies. The panel concluded that humans, including children, adult men and women, and pregnant women, have measurable

levels of unconjugated BPA in their bodies, stating succinctly that “[h]uman exposure to BPA is widespread” (vom Saal et al. 2007). Additionally, a subpanel of experts (Vandenberg et al. 2007) concluded that

Unconjugated BPA has been measured repeatedly in human blood (serum and plasma) with a central measure of the distribution in the 0.3–4.4 ng/ml range (1–19.4 nM), and in breast milk, amniotic fluid, and placental tissue in the low [nanograms per milliliter] or [nanograms per gram] range.

NTP

During the same period of time, the NTP Center for the Evaluation of Risks to Human Reproduction (CERHR) established a committee to evaluate the effects of BPA on reproductive health in humans (CERHR 2007). The original CERHR report, and several subsequent drafts, were challenged and harshly criticized by scientists because they used arbitrary criteria to evaluate animal studies, applied these criteria unevenly to different studies, and contained scientific errors and misinterpretations of published data [reviewed by Vandenberg et al. (2009)]. In the spring of 2008, the NTP undertook its own extensive review of the BPA literature, including recommendations from the CERHR report and comments from the public (NTP 2008).

The NTP (2008) limited its review to those studies related to risks for human reproduction; most of the human exposure studies available at the time were included in the assessment, whereas only a portion of the animal literature was considered useful. Regarding human exposures, the NTP came to a much less decisive conclusion compared with the Chapel Hill panel (vom Saal et al. 2007), stating that “there are data reporting bisphenol A concentrations in urine, breast milk, and amniotic fluid.” Yet, the NTP (2008) also stated that the many biomonitoring studies may be unreliable because BPA conjugates can be unstable under some storage conditions and because laboratory equipment may leach BPA: “it is possible that free bisphenol A concentrations measured in biological samples may be overestimated.” Similar to the EFSA report (EFSA 2006), the NTP (2008) reached these conclusions without evidence that contaminations had occurred.

FDA

The FDA assessed the BPA literature in 2008 (FDA 2008), stating in their assessment summary that

Based on our ongoing review, we believe there is a large body of evidence that indicates that FDA-regulated products containing BPA currently on the market are safe and the exposure levels to BPA from food contact materials, including for infants and children, are below those that may cause health effects.

The FDA (2008) largely avoided the issue of current human exposure levels, giving very little attention to either the available (> 40) biomonitoring studies or the toxicokinetic studies. The FDA (2008) summarized that

There are several publications detailing measurements in biological fluid for BPA. Although [the] FDA is aware of these data and considers them extremely useful, [the] FDA also understands the experimental limitations that have been identified with regard to these data.... [The] FDA's updated safety assessment is focused on a subpopulation, infants. Accordingly, the currently available data, which consider exposure to adults or young children (6 years of age or older), were not used or relied upon in FDA's safety assessment.

Thus, to make their decision, the FDA included no biomonitoring studies, even those from adults that clearly indicate internal exposures to unconjugated BPA (Vandenberg et al. 2010).

Biomonitoring Studies Should Be Used to Generate Risk Assessments

In our opinion, it is time to reassess how regulatory agencies such as the EFSA make decisions. Agencies should consider all available data in making risk assessments. As previously argued by Myers et al. (2009), the value of the peer-reviewed literature should not be judged on its ability to meet stringent regulatory criteria but on the strength of the integrated data. The large database of human biomonitoring data should be used to define human exposure levels and develop models for risk assessment. Studies in which humans were environmentally exposed to BPA are particularly relevant in this regard for assessing true human exposure levels, especially because BPA metabolism is influenced by age, sex, and physiological state (pregnant vs. non-pregnant) (Calafat et al. 2009; Vandenberg et al. 2007; Zalko et al. 2003). In addition, the two available toxicokinetic studies should be evaluated in their correct context, considering that *a*) their findings do not match findings from a large number of biomonitoring studies; *b*) there are serious inconsistencies in their methods and reported results; *c*) these studies are yet to be replicated; and *d*) these studies provide no information about fetal or neonatal exposure to BPA.

In summary, there is still significant controversy surrounding current human exposures to BPA. We propose that this controversy is not due to the lack of valid scientific biomonitoring studies, but instead stems from risk assessments generated using the same literature but applying different selection criteria that are not scientifically valid. We hope that the BPA saga will stimulate regulatory agencies to

reassess how they determine the usefulness of the peer-reviewed literature and lead to the use of one integrated database of scientific information, including biomonitoring studies, to protect human health.

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Urinary, Circulating, and Tissue Biomonitoring Studies Indicate Widespread Exposure to Bisphenol A

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BACKGROUND: Bisphenol A (BPA) is one of the highest-volume chemicals produced worldwide, and human exposure to BPA is thought to be ubiquitous. Thus, there are concerns that the amount of BPA to which humans are exposed may cause adverse health effects. Importantly, results from a large number of biomonitoring studies are at odds with the results from two toxicokinetic studies.

OBJECTIVE: We examined several possibilities for why biomonitoring and toxicokinetic studies could come to seemingly conflicting conclusions.

DATA SOURCES: We examined > 80 published human biomonitoring studies that measured BPA concentrations in human tissues, urine, blood, and other fluids, along with two toxicokinetic studies of human BPA metabolism.

DATA EXTRACTION AND SYNTHESIS: The > 80 biomonitoring studies examined included measurements in thousands of individuals from several different countries, and these studies overwhelmingly detected BPA in individual adults, adolescents, and children. Unconjugated BPA was routinely detected in blood (in the nanograms per milliliter range), and conjugated BPA was routinely detected in the vast majority of urine samples (also in the nanograms per milliliter range). In stark contrast, toxicokinetic studies proposed that humans are not internally exposed to BPA. Some regulatory agencies have relied solely on these toxicokinetic models in their risk assessments.

CONCLUSIONS: Available data from biomonitoring studies clearly indicate that the general population is exposed to BPA and is at risk from internal exposure to unconjugated BPA. The two toxicokinetic studies that suggested human BPA exposure is negligible have significant deficiencies, are directly contradicted by hypothesis-driven studies, and are therefore not reliable for risk assessment purposes.

KEY WORDS: endocrine disruptor, human exposure, PBPK/PBTK model, pregnancy, risk assessment, toxicokinetics. *Environ Health Perspect* 118:1055–1070 (2010). doi:10.1289/ehp.0901716 [Online 24 March 2010]

Bisphenol A (BPA) is one of the highest volume chemicals produced worldwide, with > 8 billion pounds produced each year and > 100 tons released into the atmosphere by yearly production. Data from multiple sources indicate that the amount of BPA to which humans are exposed may cause adverse health effects; this has raised concerns among regulatory agencies all over the world.

As an essential component of polycarbonate plastic, BPA is found in numerous consumer products, including baby bottles, reusable water bottles, reusable food containers, polyvinyl chloride stretch films, papers, and cardboards (reviewed by Vandenberg et al. 2007). Metallic food and beverage cans are protected from rusting and corrosion by the application of epoxy resins as inner coatings. The synthesis of many of these resins requires the condensation of BPA with epichlorohydrin to create BPA diglycidyl ether. When incomplete polymerization occurs, residual BPA leaches from the epoxy resin. High temperatures and exposure to acidic or basic solutions can also increase leaching of BPA from coatings and plastics, even when

complete polymerization has occurred. More than 10 studies have detected BPA leaching from the linings of metal cans into foods (Vandenberg et al. 2007). BPA has also been detected in a variety of environmental samples, including water, sewage leachates, indoor and outdoor air samples, and dust (Vandenberg et al. 2007). It is also found in papers and in implanted medical devices and other medical equipment (Welshons et al. 2006).

BPA attracted the attention of regulatory agencies and scientists in dozens of countries because of its estrogenic properties *in vitro* and *in vivo* and the conserved role that estrogen plays in regulating human and animal physiology and pathophysiology (Dodds and Lawson 1936; Markey et al. 2001; Wetherill et al. 2007). Biochemical assays have examined the kinetics of BPA binding to the estrogen receptors (ERs) and have determined that BPA binds both ER α and ER β , with approximately 10 times higher affinity for ER β (Gould et al. 1998; Kuiper et al. 1998). Until recently, BPA was considered a weak environmental estrogen because of its relatively low affinity for the nuclear ERs compared with estradiol in some

assays (Andersen et al. 1999; Fang et al. 2000). However, results from several studies have revealed that BPA can stimulate rapid cellular responses at very low concentrations, below the levels where BPA is expected to bind to the classical nuclear ERs (Welshons et al. 2006). BPA has also been shown to bind to a membrane-associated ER and produce nongenomic steroid actions (Wetherill et al. 2007) with the same efficacy and potency as estradiol (Alonso-Magdalena et al. 2005; Hugo et al. 2008). Whatever the mechanism, BPA can cause effects in animal models at doses in the range of human exposures, indicating that it can act at lower doses than predicted from some *in vitro* and *in vivo* assays (Richter et al. 2007; Vandenberg et al. 2007; vom Saal et al. 2007; Wetherill et al. 2007).

For risk assessment, a reference dose (RfD) is calculated as an acceptable daily human intake, typically 100-fold less than the no observed adverse effect level (NOAEL). However, the RfD for BPA (50 $\mu\text{g}/\text{kg}/\text{day}$) was calculated using the lowest observable adverse effect level (LOAEL) and 1,000-fold safety factors because a NOAEL had not been determined (Welshons et al. 2003). More than 150 published studies describe BPA effects in animals exposed to < 50 $\text{mg}/\text{kg}/\text{day}$, including altered development of the male and female reproductive tracts, organization of sexually dimorphic circuits in the hypothalamus, onset of estrus cyclicity and earlier puberty,

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altered body weight, altered organization of the mammary gland, and cancers of the mammary gland and prostate; > 40 of these studies examined doses less than the RfD (Richter et al. 2007). Many of these end points are in areas of current concern for human epidemiological trends (Soto et al. 2008; Vandenberg et al. 2009). Indeed, it has been suggested that exposure to xenoestrogens such as BPA during early development may be a major contributing factor to the increased incidence of infertility, genital tract abnormalities, obesity, attention deficit hyperactivity disorder, infertility, and prostate and breast cancer observed in European and U.S. human populations over the last 50 years (Sharpe and Skakkebaek 1993; Soto et al. 2008).

There is great concern about exposure of human fetuses, infants, and neonates to BPA because of the sensitivity of the developing organs and brain to exogenous hormones (Vandenberg et al. 2009). However, for translating the findings from animal studies to health risks of environmental exposures to humans, exposure assessment from biomonitoring of BPA in different populations is essential.

In this review, we examined > 80 biomonitoring studies that measured BPA concentrations in human tissues and fluids, specifically focusing on individuals that were exposed to BPA via their environment (i.e., non-occupational exposures). These studies, examining thousands of individuals from several different countries, overwhelmingly detected BPA in individual adults, adolescents, and children. Interestingly, results from the large body of research encompassing biomonitoring studies are at odds with the results from two toxicokinetic studies that determined the disposition of BPA in humans after oral administration of BPA (Volkel et al. 2002, 2005).

Volkel et al. (2002) reported that after oral absorption, BPA was promptly metabolized in the liver and intestines. However, findings from the > 80 biomonitoring studies we reviewed support the hypothesis that hepatic metabolism of BPA or its presystemic clearance is not 100% efficient, because unconjugated BPA has been regularly detected in urine and blood samples. Theoretically, the presence of unconjugated BPA in blood and/or urine samples could also be explained by exposure via non-oral routes that circumvent liver and intestinal first-pass metabolism (Stahlhut et al. 2009). All sources for oral and non-oral exposures have not been definitively identified, thus increasing the value of biomonitoring studies, which do not take into account exposure sources. Throughout this review, when we discuss conjugated BPA it is specifically identified; otherwise, "BPA" refers to the unconjugated molecule. Note also that when we identify significant differences, we

are referring to statistically significant differences identified by the original study authors, each of whom established a relevant α -value for their study populations.

In this review, we propose several hypotheses to explain why biomonitoring and toxicokinetic studies could come to seemingly conflicting conclusions. Additionally, the reliance of regulatory agencies on the two studies that predict no human exposure, in contrast to the > 80 studies that measure actual internal exposures, is contrary to scientific principles.

Biomonitoring Studies: General Overview

Biomonitoring studies allow the determination of internal circulating levels and excreted concentrations of a chemical of interest, which account for exposures from all possible sources, rather than suspected exposures from specific sources. In the United States, the Centers for Disease Control and Prevention (CDC) is the major source for information on human exposures to a multitude of environmental contaminants and has developed sensitive assays to reliably measure them (CDC 2008; Kuklenyik et al. 2009). In Europe, the German Environmental Survey is a representative population study to determine the exposure of Germany's general population to environmental contaminants (Umwelt Bundes Amt 2009). Its main objective is to generate, update, and evaluate representative data in order to facilitate environmental health-related observations and reporting of information at the national level.

Proper biomonitoring studies take into account the kinetics, bioaccumulative properties, and metabolism of the target chemical to determine which tissue (biological matrix) should be examined. The chemical properties of the substance being examined also have an impact on the matrices that can be examined reliably, because some substances are altered by enzymes in blood and other substances can break down in urine (Calafat and Needham 2008). Furthermore, the toxicokinetics of the substance being investigated is likely to be influenced by the physiological status of the individual. Finally, the sensitivity and reliability of the methods used for analysis, the collection methods and materials, and the contamination of laboratory chemicals and equipment with the substance of interest are all important factors to be considered in biomonitoring studies. We discuss these issues in greater detail below.

Analytical Methods Used in Biomonitoring Studies

Multiple techniques have been used to measure total, unconjugated, and conjugated BPA in human blood, urine, and tissue samples. Gas chromatography (GC) and liquid

chromatography (LC) are typically used with various detection methods, including mass spectrometry (MS), tandem MS (MS/MS), and electrochemical or fluorescence derivatization. The CDC has measured BPA using solid-phase extraction coupled with isotope dilution-HPLC (high-performance liquid chromatography)-MS/MS, which is considered the "gold standard" for urine biomonitoring studies because of its high level of accuracy, negligible interference, and ability to identify chemical structures (Calafat et al. 2008). However, this method is limited by its high cost per sample, making it impractical for many studies.

The methods used in biomonitoring studies are crucial for the acceptance of the results (Dekant and Volkel 2008; Vandenberg et al. 2007). The ELISA (enzyme-linked immunosorbent assay) has also been used in several studies of human fluids and tissues because it is convenient, inexpensive, and useful for the screening of a large number of samples. However, the use of ELISA to measure BPA concentrations in human samples has been specifically challenged because this method is considered less specific than methods employing analytical chemistry (Dekant and Volkel 2008; Fukata et al. 2006); that is, there is concern that ELISA assays detect substances other than BPA and its conjugates, including other bisphenols (Ohkuma et al. 2002). Studies rarely report the controls that are necessary to determine whether or not cross-reactivity has occurred; however, if information about cross-reactivity and standard validations are included, these data should be considered valid, as they are for the many other compounds that are measured with this method. In general, ELISA has fallen out of favor for the quantification of BPA in human biomonitoring studies, particularly because more sensitive, accurate, and precise methods employing analytical chemistry are now available for high-throughput screens (Kuklenyik et al. 2009). Nonetheless, it should be recognized that this method is much more affordable than analytical chemistry methods and, most important, it has successfully been used to measure BPA in samples in a comparable dose range and in a similar percentage of samples as the methods that are considered more accurate (Vandenberg et al. 2007).

In addition to the methods used to measure BPA in human tissues and fluids, the equipment and containers used to collect and store samples are critical for the accurate assessment of BPA concentrations. It has been suggested that the low levels of unconjugated BPA detected in bodily tissues and fluids were due to contamination from collection materials or nonenzymatic deconjugation of BPA during storage (Atkinson et al. 2002; Dekant and Volkel 2008; Willhite

et al. 2008). Organic solvents used in the laboratory may release BPA from plastic labware, leading to contamination; water has also been found to contain detectable levels of BPA (Vandenberg et al. 2007), and the columns used for HPLC may contain and/or trap BPA (Volkel et al. 2005). For these reasons, the use of adequate blanks is essential for every step of the biomonitoring process, including sample collection and every point in the chemical analysis. These blanks should be subjected to the same extraction/injection protocols as the actual samples. Most of the biomonitoring studies we reviewed included data from blanks to indicate that, contrary to the claims made by some scientists (Dekant and Volkel 2008), contamination from equipment cannot explain the concentrations of BPA reported.

In terms of sample collection, studies should control for contamination from syringes used to draw samples, pipettes used to transfer plasma/serum to storage tubes, and the nature of sample storage tubes. If samples are obtained in hospital settings where the patient has an intravenous tube, it is essential to account for BPA leaching from these tubes during saline infusion. Studies need to be performed using appropriate matrix (blood or urine) spiked with known amounts of BPA and subjected to collection procedures similar to that of actual sampling to rule out BPA contribution from these various sources. One study used sheep blood to perform such validations and found very little BPA contamination from these sources (Padmanabhan et al. 2008). However, for translation to humans, similar studies using human blood and/or urine need to be undertaken.

Ye et al. (2007) addressed the issue of sample storage by comparing the stability of BPA conjugates for urine stored for up to 6 months at three temperatures: room temperature, 4°C, or -70°C. Storage at -70°C kept conjugated BPA stable for at least 180 days, whereas storage at room temperature led to degradation of BPA conjugates within 24 hr. The effects of storage temperature on the detection of BPA and BPA conjugates in saliva samples have also been investigated. Atkinson et al. (2002) spiked saliva samples with BPA, BPA dimethylacrylate (Bis-DMA), or triethylene glycol dimethacrylate (TEGDMA), stored at -20°C or -70°C, and then tested the samples by HPLC and GC-MS. In contrast to samples stored at -20°C, concentrations of BPA, Bis-DMA, and TEGDMA were unchanged in salivary samples stored at -70°C, suggesting that this condition was appropriate for biological sample storage. Finally, in a recent study Ye et al. (2009b) examined the stability of phenols in 16 commercially available serum samples stored under worst-case-scenario conditions

(4°C, 25°C, or 37°C). Concentrations of BPA metabolites and other phenols did not vary significantly, even when samples were stored at 37°C for at least 30 days. This finding suggests that phenolic compounds, likely including BPA, are more stable in serum than in urine, and it gives greater credence to those studies that measured BPA in human serum samples.

Exposure Assessment from Urinary Measures of BPA

In 2001, the CDC conducted the first biomonitoring study of BPA (unconjugated and conjugated) in pooled urine samples (Brock et al. 2001). The authors developed a method that was fairly sensitive [limit of detection (LOD) = 0.12 ng/mL]; concentrations of unconjugated BPA were < LOD, and concentrations of BPA glucuronide ranged from 0.11 to 0.51 ng/mL. After this initial examination in pooled urine, more than a dozen additional small studies examined urinary BPA concentrations and/or its metabolites in < 100 adults each (Arakawa et al. 2004; Carwile et al. 2009; García-Prieto et al. 2008; Joskow et al. 2006; Kawaguichi et al. 2005; Kim et al. 2003; Liu et al. 2005; Mao et al. 2004; Matsumoto et al. 2003; Moors et al. 2007; Nepomnaschy et al. 2009; Ouichi and Watanabe 2002; Schöringhumer and Cichna-Markl 2007; Tsukioka et al. 2003; Volkel et al. 2005; Yang et al. 2003; Ye et al. 2005a, 2005b) (Table 1). Although these studies used slightly different methods and different population samples, they overwhelmingly detected BPA or its conjugates in urine. Collectively, these studies examined spot urine samples collected from 604 adults, and BPA and/or its conjugates were detected in 518 (85.8%).

Because most studies of urine used enzymatic treatment with either glucuronidase or sulfatase, or both, many studies reported only the total BPA concentration, that is, unconjugated plus conjugated BPA. Of the seven studies that specifically examined unconjugated BPA, six detected unconjugated BPA in at least one sample (Calafat et al. 2009; Kim et al. 2003; Ouichi and Watanabe 2002; Schöringhumer and Cichna-Markl 2007; Volkel et al. 2008; Ye et al. 2005b); only one study failed to detect unconjugated BPA in any sample (Brock et al. 2001). The distinction between conjugated and unconjugated BPA is an important one, especially in blood samples, where the presence of unconjugated BPA in circulation indicates internal exposure to the parent compound, which is estrogenically active. The presence of unconjugated BPA in urine is also indicative of internal exposure to BPA and suggests either a failure of first-pass metabolism to conjugate and rapidly remove BPA from the body or the deconjugation of BPA metabolites in the body. It is

also possible that these repeated measurements of unconjugated BPA in urine are the result of nonenzymatic deconjugation of conjugated BPA during storage, although storage protocols provided in these studies and its repeated detection suggest that this is unlikely.

In contrast to the many small studies detecting BPA and/or its conjugates in urine, one study failed to detect either unconjugated or conjugated BPA in the 19 samples examined (Volkel et al. 2005). To our knowledge, this is the only published study that completely failed to detect or measure any form of BPA in urine samples from environmentally exposed individuals. Volkel et al. (2005) used HPLC-MS/MS; this is the same technique that was used in several other studies in which unconjugated and conjugated BPA were detected in urine (Calafat et al. 2008; Hong et al. 2009; Mahalingaiah et al. 2008; Nepomnaschy et al. 2009; Teitelbaum et al. 2008; Wolff et al. 2007; Yang YJ et al. 2009; Ye et al. 2009a). Why did Volkel et al. (2005) not detect any form of BPA? The first possibility to consider is whether their study was the only one that successfully avoided external contamination of samples with BPA. Because several of these biomonitoring studies were performed by high-standard analytical laboratories (e.g., CDC), it is unlikely that all of these studies except the one by Volkel et al. (2005) failed to adequately prevent external contaminations (Ginsberg and Rice 2009). The second possibility to consider is whether Volkel et al. (2005) used a method with sufficient sensitivity to detect any form of BPA in urine. Unlike the other studies using HPLC-MS/MS, which is generally a very sensitive method (LODs ≤ 0.4 ng/mL), the LOD of Volkel et al.'s study was 1.14 ng/mL. Two other studies used analytical methods with similar or higher LODs: Matsumoto et al. (2003) used HPLC and detected total BPA in most of the 106 individuals examined (LOD = 1.7 ng/mL), and Mao et al. (2004) used HPLC with fluorescence detection and found total BPA in most of the 20 individuals examined (LOD = 2.7 ng/mL). In addition, considering the mean (or median) concentrations of total BPA detected in other studies examining urine samples with HPLC-MS/MS [2.0 ng/mL (Wolff et al. 2007); 2.6 ng/mL (Calafat et al. 2008); 1.3 ng/mL (Mahalingaiah et al. 2008); 2.7 ng/mL (Hong et al. 2009); 1.82 ng/mL (Nepomnaschy et al. 2009); 0.52–0.61 ng/mL (Yang YJ et al. 2009); 2.52–4.50 ng/mL (Ye et al. 2009a)], this less sensitive analytical method would likely be able to detect some form of BPA in the average person. To address the issue of whether their experimental system was able to detect BPA at all in urine samples, Volkel et al. (2005) spiked urine samples with large amounts of BPA glucuronide. Under these conditions, they

were able to successfully detect this compound using their methodology, suggesting that there were no problems detecting high concentrations of BPA metabolites in urine. Considering that this study examined < 20 adults, the negative result reported by Volkel et al. (2005) could be explained as a sampling error combined with a relatively insensitive analytical method, but this is unlikely, leaving us with no scientific explanation for their lack of detection of BPA. Because science is based on repeatability of data, the fact that this one negative study stands against more than a dozen studies that successfully measured BPA in urine samples suggests that it is an outlier.

The smaller studies described above provide little information about exposure of the

general population. In 2005, the first study to measure total BPA concentrations in a reference population was performed by the CDC (Calafat et al. 2005); total BPA was detected in 95% of spot urine samples collected from 394 American adults. The CDC followed this study with a second one, examining spot urine samples from 2,517 Americans > 6 years of age from the 2003–2004 National Health and Nutrition Examination Survey (NHANES) (Calafat et al. 2008). Total BPA was detected in 92.6% of participants, with concentrations ranging from 0.4 to 149 ng/mL and a geometric mean (GM) of 2.6 ng/mL urine.

Urinary volume is influenced by various factors (i.e., glomerular filtration, tubular secretion and reabsorption, alimentary regimen,

intake of liquids, perspiration) that give rise to differing urinary dilutions and, consequently, to changes in the concentrations of excreted substances (Carrieri et al. 2000). Dilute or concentrated samples provide low or high values, respectively, of the biological marker, with underestimation or overestimation of the result. Therefore, to compare exposures among groups of individuals, urinary concentrations of chemicals must be adjusted to urinary creatinine concentration (Barr et al. 2005). After correcting for urine creatinine concentration, Calafat et al. (2008) found that total urinary BPA concentrations were associated with age, sex, race/ethnicity, and household income.

Four additional studies examined fairly large populations of adults. In a study of

Table 1. BPA levels in human urine after environmental exposures.

Reference	Detection method and enzymatic treatment ^a	LOD (ng/mL)	Sample size	Study population	Detection rate	BPA level [ng/mL (ppb), mean ± SE]	
						Unconjugated BPA	Total BPA
Brook et al. 2001	GC-MS ^b	0.12	Five pools	Each pool contained > 5 individuals	5/5 pools	All < LOD	Range, 0.1–0.51
Ouchi and Watanabe 2002	HPLC-ECD with column switching ^b	0.2	48	Female college students (Japan)	2% unconjugated BPA, 100% BPA glucuronide	Range, ND–0.2 ^c	
Kim et al. 2003	RP-HPLC-FD	0.28	15	Korean males	100% unconjugated and total BPA	0.58 ± 0.14 ^{c,d}	2.82 ± 0.73
				Korean females		0.56 ± 0.10 ^{c,d}	2.76 ± 0.54
Matsumoto et al. 2003	HPLC	1.7	50	College students in 1992	82%		Exact values NR
				College students in 1999	61%		Exact values NR
Tsukiyama et al. 2003	NCI-GC-MS ^b	0.1	6	Japanese adults	100%		Mean, 1.6
Yang et al. 2003	HPLC-FD ^b	0.012	73	Koreans with various <i>SULT1A1</i> polymorphisms	75%		Mean, ~ 9.5
Arakawa et al. 2004	GC-MS/MS ^b	0.36	36	Japanese males	100%		Range, 0.2–14 (ug/day)
Mao et al. 2004	HPLC-FD ^e	2.7	10	Chinese males	60%		Range, ND–395
				Chinese females	100%		Range, 3–374
Calafat et al. 2005	GC-MS ^b	0.1	184	American males	95%		GM, 1.63
				American females	94%		GM, 1.12
Kawaguchi et al. 2005	SBSE-GC-MS	0.02	5	Adult volunteers	80%		Range, ND–5.41
Liu et al. 2005	HPLC with ECD ^b	0.5	9	American girls, 9 years of age	89%		Median, 2.4
				American adults	52%		Median, 0.47
Volkel et al. 2005	HPLC-MS/MS ^b	1.14	6	German adults	0%	— ^c	< LOD
Ye et al. 2005a	Online SPE	0.4	30	American adults	87%		GM, 3.5
Ye et al. 2005b	Online SPE	0.3	30	American adults	97%	Mean, < LOD ^{c,d}	Mean, 3.2
Martin et al. 2005	SPE-HPLC-MS/MS ^b	NR	19	Children before dental treatment; ages not given	NR		GM, 0.26
Joskow et al. 2006	GC-MS ^b	0.1	14	Men before dental treatment	NR		2.41 ± 0.33
Yang et al. 2006	HPLC-FD ^b	0.026	172	Koreans with various <i>SULT1A1</i> polymorphisms	97.5%		Median, 7.86
Moors et al. 2007	GC-MS	3	15	German adults	60%		Range, ND–55
Schönghuber and Gichna-Markl 2007	SGIC-HPLC-FD	0.2	12	Austrian adults	75% unconjugated BPA, 100% total	Median, 0.3	Median, 1.1
				10	Dialysis patients (Austria)	90% unconjugated BPA, 100% total	Median, 0.2
Wolff et al. 2007	HPLC-MS/MS ^b	0.36	90	American girls, 6–9 years of age	94%		GM, 2.0
Calafat et al. 2008	HPLC-MS/MS	0.4	314	Children, 6–11 years of age	92.6% of all individuals examined		GM, 4.3
			713	Adolescents, 12–19 years of age		GM, 2.8	
			950	Adults, 20–59 years of age		GM, 2.4	
			537	Adults > 60 years of age		GM, 2.3	
García-Prieto et al. 2008	CME-LC-FD ^b	0.197	8	Spanish college students	25% unconjugated BPA, 100% total		Range, 4.03–49

continued next page

172 Korean adults, Yang et al. (2006) found that 97.5% had detectable levels of total BPA, and in a study of 516 Korean adults, Hong et al. (2009) found detectable levels of total BPA in 76%. Yang YJ et al. (2009) examined urine from 485 Korean adults (259 men, 92 premenopausal women, and 134 postmenopausal women) and found detectable levels of total BPA in 76%, a strikingly similar result. In the fourth study, Volkel et al. (2008) examined 438 urine samples from 285 individuals, as well as additional samples collected from members of the research team; some of the samples examined had been stored for several years at -20°C . Because of problems with methodology, total BPA could only be

measured in a subset of the samples; total BPA concentrations ranged from undetectable levels to 9.3 ng/mL. In sum, these six large-scale studies (Calafat et al. 2005, 2008; Hong et al. 2009; Volkel et al. 2008; Yang et al. 2006; Yang YJ et al. 2009) reinforce findings from small studies, providing further evidence that most individuals in several countries have detectable levels of BPA and/or its conjugates in their urine (Table 1).

Levels of BPA exposure and concentrations in bodily fluids and tissues from individuals in less developed countries have been relatively understudied and were previously identified as an important research need (Vandenberg et al. 2007). In a recent study in

China, He et al. (2009) addressed this issue by examining > 900 human subjects. Detectable levels of total BPA were measured in 50% of the subjects. With this large sample size, the authors were able to detect several statistically significant associations: Detection rates were higher in males, in people > 40 years of age, in people with more education, and in individuals who smoked and/or consumed alcohol. Some of these results differ from the CDC's findings in the United States (i.e., sex- and age-related differences in exposure) and thus may suggest that exposure routes or sources are different between these populations.

Biomonitoring measurements for adults are generally not informative about internal

Table 1. continued

Reference	Detection method and enzymatic treatment ^a	LOD (ng/mL)	Sample size	Study population	Detection rate	BPA level (ng/mL (ppb), mean \pm SE)	
						Unconjugated BPA	Total BPA
Mahalingaiah et al. 2008	HPLC-MS/MS	0.4	45	Women in a fertility clinic	87% of all samples	Range, ND–42.6; GM, 1.09	
			37	Men in a fertility clinic		Range, ND–18.7; GM, 1.62	
Teitelbaum et al. 2008	HPLC-MS/MS	0.36	159 samples from 35 children	American children, 6–10 years of age	95%	GM, 3.4	
Volkel et al. 2008	Online SPE	0.3	31	German women	NR for any samples or groups	ND–2.5	
	HPLC-MS/MS ^b		30	German children, 5–6 years of age		ND–0.9	
			315 samples from 203 subjects	Archived samples collected from adults (2005)		All < LOQ (5 ng/mL)	
			62 samples from 21 subjects	Laboratory workers (Germany)		ND–1.8	
Wolff et al. 2008	Online SPE-HPLC-MS/MS	0.36	404	Pregnant American women	90.8%	Range, ND–35.2	
Ye et al. 2008b	GC-MS/MS ^c	0.25	100	Pregnant Dutch women	82%	Median, 1.2	
Becker et al. 2009	LC/LC-MS/MS	0.25	137	German children, 3–5 years of age	99% of all samples > LOQ	GM, 3.55	
			145	German children, 6–8 years of age		GM, 2.72	
			149	German children, 9–11 years of age		GM, 2.22	
			168	German adolescents, 12–14 years of age		GM, 2.42	
Calafat et al. 2009	Online SPE-HPLC-MS/MS	0.4	419	Premature American infants	92% unconjugated BPA, 100% total	Range, ND–17.3; median, 1.7; GM, 1.8	Range, 1.6–946; median 28.6; GM, 30.3
Carwile et al. 2009	Online SPE-HPLC-MS/MS	0.4	77	American college students avoiding polycarbonate bottles	88%	GM, 1.3	
				Same students using polycarbonate bottles	96%	GM, 2.1	
He et al. 2009	HPLC ^d	0.31	419	Chinese males	58%	GM, 1.41	
			503	Chinese females	44%	GM, 0.58	
Hong et al. 2009	HPLC-MS/MS	0.063	516	Korean adults	76%	2,742 \pm 0.39	
Nepomnaschy et al. 2009	HPLC-MS/MS ^e	0.18	60	Premenopausal women	100%	1.82 \pm 0.33	
Yang YJ et al. 2009	HPLC-MS/MS	0.0063	259	Korean men	75%	GM, 0.52	
			92	Premenopausal Korean women	80%	GM, 0.61	
			134	Postmenopausal Korean women	75%	GM, 0.58	
			10 pools	Pregnant Norwegian women	NR for any samples or groups	GM, 4.50	
Ye et al. 2009a	HPLC-MS/MS	0.26	110	Pregnant Dutch women		GM, 2.52	
			87	Pregnant American women		GM, 3.93	

Abbreviations: CME, coextractive microextraction; ECD, electron capture detection; FD, fluoremetric detection; GM, geometric mean; LOQ, limit of quantification; NCI, negative chemical ionization; ND, not detected; NR, not reported; RP, reverse phase; SBSE, stir bar sorptive extraction; SGIC, sol-gel immunoaffinity column; SPE, solid-phase extraction; *SULT1A1*, sulfotransferase 1A1 gene.

^aAll samples were treated with glucuronidase and sulfatase unless otherwise noted. ^bSamples were treated with glucuronidase only. ^cBPA glucuronide was also measured. ^dBPA sulfate was also measured. ^eSamples were treated with chemical hydrolysis, which deconjugates both sulfate and glucuronide groups. ^fCorrected for creatinine and presented as $\mu\text{g/g}$ creatinine. ^gOnly 37 samples were tested for unconjugated BPA.

concentrations or exposure levels in children. The CDC study (Calafat et al. 2008) examined total BPA content in spot urine samples from 314 American children 6–11 years of age and reported a GM concentration of 3.6 ng/mL urine. The GM level of total BPA in the 715 adolescents 12–19 years of age examined in the same study was 3.7 ng/mL urine. When adjusted for creatinine levels, exposures were highest in children and still significantly higher in adolescents than in adults. Six additional studies measured BPA and its metabolites in urine of infants and children. Liu et al. (2005) examined urine from nine 9-year-old American girls and detected total BPA in eight of the girls. Wolff et al. (2007) detected total BPA in 94% of urine samples collected from 90 American girls 6–9 years of age. In another study, Teitelbaum et al. (2008) examined 159 urine samples that had been collected from 35 black and Hispanic American children 6–10 years of age; of these spot urine samples, 95% had detectable concentrations of total BPA, with a GM of 3.4 ng/mL urine, a level very similar to those of children examined in the larger CDC study (Calafat et al. 2008). In a large study that included a subset of 30 German children 5–6 years of age, Volkel et al. (2008) measured unconjugated BPA concentrations up to 0.9 ng/mL and total BPA concentrations up to 7.5 ng/mL, although the frequency of detection was not reported. In a study of 599 German children 3–14 years of age, Becker et al. (2009) detected total BPA in the urine of 591 individuals (98.7%; GM of 2.66 ng/mL); they detected significantly higher levels in children 3–5 years of age (GM, 3.55; $n = 137$). Finally, Calafat et al. (2009) studied 41 premature infants in an American neonatal infant care unit and found BPA conjugates in the urine of all the babies examined. Amazingly, the GM concentrations measured in these infants were 11 times higher than the concentrations measured in the NHANES study (30.3 vs. 2.6 ng/mL urine). Unconjugated BPA was also detected in the urine of 34 of the 37 neonates examined, with a GM of 1.8 ng/mL urine (Calafat et al. 2009). These authors noted that the presence of unconjugated BPA in urine was unexpected, and because of the collection of urine from cotton placed in diapers, there is uncertainty as to whether this is a contaminant from the collection process or a true measure of unconjugated BPA from these young individuals. Collectively, these seven studies included 41 premature infants (Calafat et al. 2009), 909 children 3–11 years of age (Becker et al. 2009; Calafat et al. 2008; Liu et al. 2005; Teitelbaum et al. 2008; Volkel et al. 2008; Wolff et al. 2007), and 883 adolescents 12–19 years of age (Becker et al. 2009; Calafat et al. 2008) and repeatedly found detectable levels of total BPA in a vast majority of the

individuals examined. These studies clearly indicate that children are exposed to BPA and suggest that exposures are highest among neonates and young children.

Four studies examined total BPA concentrations in urine collected from women during pregnancy, further suggesting exposure of the fetus to BPA during gestation (Table 1). First, in a study examining spot urine samples collected from 100 pregnant Dutch women, Ye et al. (2008b) detected total BPA in 82% of samples, with a median concentration of 1.2 ng/mL urine, similar to the levels of BPA detected in other populations, including U.S. reference populations (Calafat et al. 2005, 2008). An even larger study examined urine from 404 American women in their third trimester of pregnancy. In that study Wolff et al. (2008) detected total BPA in the urine of 90.8% of the women, and urine concentrations were associated with offspring birth weight. A third study examined total BPA in 10 pooled urine samples from 110 pregnant Norwegian women (Ye et al. 2009a); the concentrations measured were higher than in any other study of pregnant women (mean, 4.5 ng/mL). These authors also examined urine samples from 110 pregnant Dutch women and 87 pregnant American women. The concentrations in these populations were relatively high compared with other groups that have been examined, with mean concentrations of 2.52 and 3.93 ng/mL, respectively (Ye et al. 2009a). In the final study to include urine samples from pregnant women (Mahalingaiah et al. 2008), samples were collected from a fertility center in Massachusetts. In urine samples collected from 45 women before pregnancy, the GM concentration of total BPA was 1.09 ng/mL urine. During the study period, 10 subjects became pregnant. In these women, total BPA concentrations in their urine were 26% higher than in urine of nonpregnant women and 33% higher than in urine samples collected before pregnancy. This last finding is perhaps the most concerning indicator of exposure of the human fetus to BPA.

Other studies have estimated associations between BPA concentrations and activities that may increase BPA exposure. For example, Matsumoto et al. (2003) associated the urinary concentrations of Japanese subjects with their consumption of coffee and tea drinks from cans containing BPA resins. Interestingly, when can coatings were changed to a formula with a lower BPA content, the association they found was no longer evident; in addition, removal of BPA from can linings led to a > 50% reduction in conjugated BPA detected in urine. Carwile et al. (2009) measured total BPA concentrations in urine collected from a group of American college students during a washout phase when all cold beverages

were consumed from stainless steel containers, and compared these concentrations with those from the same students during a phase when all cold beverages were consumed from polycarbonate plastic containers. The authors found that urinary concentrations of total BPA were increased significantly (69% higher) during the week when polycarbonate bottles were used, suggesting that these containers may be a significant source of BPA exposure to individuals in this age group and that interventions would help lower exposure levels. Joskow et al. (2006) made an interesting observation about urinary concentrations of total BPA in male volunteers receiving dental sealant treatments: Compared with baseline levels (2.41 ng/mL), individuals who received one brand of sealant known to contain BPA had significantly more BPA metabolites in their urine 24 hr after sealant placement (7.34 ng/mL), whereas individuals who received a different sealant had concentrations similar to the pretreatment level (2.06 ng/mL) (Joskow et al. 2006). Another brief study examined total BPA in urine samples collected from American children before and after placement of dental sealants (Martin et al. 2005). Total BPA levels increased rapidly from baseline levels (0.26 ng/mL) within 24 hr of sealant placement (1.18 ng/mL) and peaked 7 days after placement (1.21 ng/mL). However, the measured concentrations did not return to baseline 14 days after placement (0.73 ng/mL), suggesting that exposure of children to BPA from dental sealants may be more than the acute exposure that has been suspected. Finally, Schöringhumer and Cichna-Marik (2007) compared urinary concentrations of unconjugated and total BPA in 10 dialysis patients and 12 healthy adults but found no significant differences between these two groups.

Estimates of BPA Consumption from Urinary Measures

Only a few studies have estimated total BPA exposure in the human population. Using exposure estimates from a variety of environmental sources (i.e., water, air, and soil) and from food and beverage contamination (i.e., leaching rates from plastic containers and can linings), several studies estimated daily human intake of < 1 µg/kg body weight (BW)/day (Kang et al. 2006; Wilson et al. 2003, 2007). Additional studies have estimated daily BPA exposure by calculating BPA ingestion from food sources (European Union 2002). Biomonitoring data provide more reliable information about exposures because they do not require all the sources of exposure to be identified. This is especially important for the case of BPA, where non-oral routes of exposure are suspected and the totality of sources has not yet been identified (Stahlhut et al. 2009; Welshons et al. 2006).

Using toxicokinetics, urinary BPA levels have been extrapolated to estimate daily intake levels. For instance, using urine samples collected from 48 women, Ouchi and Watanabe (2002) estimated daily intake to be 0.6–71.4 $\mu\text{g}/\text{day}$. Using backward calculations from urinary BPA concentrations detected in Japanese adults, worst-case-scenario daily intakes for that population were estimated at 0.037–0.064 $\mu\text{g}/\text{kg BW}/\text{day}$ for males and 0.043–0.075 $\mu\text{g}/\text{kg BW}/\text{day}$ for females (Miyamoto and Kotake 2006). Kamrin (2004) utilized urine concentrations reported in two previous studies (Arakawa et al. 2004; Broćk et al. 2001) to calculate daily intake levels of 0.002–0.3 $\mu\text{g}/\text{kg BW}/\text{day}$. Although these calculations and estimates have provided a range of doses that researchers can target for experimental animal studies, these figures were determined based on toxicokinetic models that may be flawed, a topic that we discuss in further detail below.

Salivary Measures of BPA

Like urine, saliva is a preferred bodily fluid for biomonitoring purposes because collection requires relatively noninvasive procedures. To date, studies that have examined saliva for BPA have focused on the effects of dental sealant application to BPA concentrations. Since the 1960s, BPA diglycidyl methacrylate has been used as a component of many dental restorative materials, including those used for sealing molars.

Six studies have measured BPA in saliva after dental sealant application, and all were able to detect BPA in the saliva of some of the individuals examined (Arenholt-Bindslev et al. 1999; Fung et al. 2000; Joskow et al. 2006; Olea et al. 1996; Sasaki et al. 2005; Zafra et al. 2002) (Table 2). These studies used different analytical methods and examined

saliva collected at different points after sealant application. Although these studies provide interesting information about the dynamics of BPA leaching from sealants shortly after sealant placement (Vandenberg et al. 2007), they are less informative about the use of saliva as a matrix for biomonitoring of BPA. Joskow et al. (2006) measured BPA concentrations in saliva before any treatment, with a mean level of 0.3 ng/mL saliva; this concentration is much lower than that measured in urine (Table 1). Owing to the possibility of contamination with BPA leaching from dental materials, saliva does not seem to be a reliable biomonitoring tool for estimating systemic exposure to unconjugated BPA.

Internal Dosage Assessment from Blood Measures of BPA

Seventeen studies have measured BPA in blood and serum samples from healthy male and nonpregnant female adults (Dirtu et al. 2008; Fung et al. 2000; He et al. 2009; Hiroi et al. 2004; Ikezuki et al. 2002; Inoue et al. 2000, 2001; Kaddar et al. 2009; Kuroda et al. 2003; Liu et al. 2007; Sajiki et al. 1999; Sugiura-Ogasawara et al. 2005; Takeuchi and Tsutsumi 2002; Takeuchi et al. 2004; Volkel et al. 2005; Yang M et al. 2009; Yoshimura et al. 2002) (Table 3). Of these studies, five used ELISA, one used a radioimmunoassay (RIA), and the remainder used analytical chemistry to assess BPA concentrations. These studies had similar LODs compared with studies of urine, but unlike most urine studies, measurements performed on blood, serum, or plasma samples usually measured unconjugated BPA specifically. In healthy, nonpregnant adults, unconjugated BPA was detected in 14 of 16 studies (including 8 of 10 studies using analytical chemistry). Compared with the magnitude of studies examining BPA levels in urine, most

of these studies involved smaller sample sizes. Large-scale biomonitoring studies measuring circulating levels of unconjugated BPA, an index of internal exposure levels, remain to be done, particularly in the United States; only two studies to date examined > 100 individuals, and neither of these included samples from Americans. However, of those studies that used analytical chemistry methods and measured detectable levels of BPA, mean concentrations were typically in the range of 1 ng/mL blood. These concentrations are quite similar to those reported for BPA conjugates in urine (Table 1) and clearly indicate that humans are internally exposed to unconjugated BPA.

In one of the larger studies of human blood, Kaddar et al. (2009) examined plasma samples from 207 individuals, collected randomly in a French hospital, using an RIA (LOD = 0.08 ng/mL plasma; correlation with HPLC-MS, $r^2 = 0.92$). Unconjugated BPA was detected in 83% of the samples, and 12% had > 2 ng/mL plasma. Patients undergoing dialysis were also examined in this study, and > 70% of patients had measurable concentrations of unconjugated BPA > 10 ng/mL plasma. In another recent study performed in China, He et al. (2009) measured total BPA concentrations in > 900 individuals. Because samples were treated with β -glucuronidase, it is impossible to determine the proportion of conjugated and unconjugated BPA that was present. Measurable levels of total BPA were found in 17% of the samples; detection levels were significantly higher in females than in males and in people < 40 years of age compared with people > 40 years of age, and were highest in individuals who smoked and/or consumed alcohol. Finally, a study examining stored blood samples collected from Korean women during 1994–1997 measured both unconjugated and total BPA

Table 2. BPA levels in saliva.

Reference	Detection method	LOD (ng/mL)	Sample size	Sample	End point(s)	Leaching level ($\mu\text{g}/\text{mL}$)
Olea et al. 1996	HPLC (verified by GC-MS)	NR	18	Patients with 50 mg of sealant applied to a total of 12 molars	Saliva 1 hr after application	Range, 3.3–30
Arenholt-Bindslev et al. 1999	HPLC	100	8	Patients with a total of 38 mg of sealant applied to a total of 4 molars	Saliva immediately after application Saliva 1 hr after application Saliva 24 hr after application	Range, ND–2.8; mean, 1.43 Undetected in any sample Undetected in any sample
Fung et al. 2000	HPLC-FD	5	22	Patients with 32 mg of sealants applied to a total of 4 molars	Saliva 1–3 hr after application	Range, 0.0058–0.1056
Zafra et al. 2002	GC-MS	3	8	Patients undergoing dental repairs	Saliva 1 hr after application	Range, 0.0153–0.0324
Sasaki et al. 2005	ELISA	NR	21	Patients treated with one of nine resins	Saliva after application and gargling	Range, 0.0210–0.0601
Joskow et al. 2006	GC-MS	0.1	14	Patients treated with one of two dental sealants (Delton and Helioseal)	Saliva before dental sealant application Saliva immediately after Delton sealant application Saliva 1 hr after Delton sealant application Saliva immediately after Helioseal sealant application Saliva 1 hr after Helioseal sealant application	0.00030 \pm 0.000043* 0.0428 \pm 0.01032 0.00786 \pm 0.00424 0.00054 \pm 0.00020 0.00021 \pm 0.000013

Abbreviations: FD, fluorometric detection; ND, not detected; NR, not reported.
*Values are mean \pm SE.

in > 150 individuals (Yang M et al. 2009). About half of these women were breast cancer patients, and the other half were age-matched controls. These authors found no associations between breast cancer status and BPA concentrations. Total BPA was detected in 50.8% of the samples. However, the levels of unconjugated BPA in most samples were < LOD (0.012 ng/mL).

We are aware of only two studies that were unable to detect BPA in any individual samples of blood from adults (Fung et al. 2000; Volkel et al. 2005). Fung et al. (2000) examined 40 adults before and after the application of dental sealant materials containing

BPA. This study had an LOD of 5 ng/mL; considering other studies that suggest exposures are typically in the 1 ng/mL range, it is not surprising that this study was unable to detect BPA in blood. In the second study that did not detect BPA in the blood of healthy individuals, Volkel et al. (2005) used LC-MS/MS, typically a highly sensitive method, but examined only 19 adults. The authors of that study reported multiple LODs for blood samples (0.57 and 1.14 ng/mL), although a later study from this group suggested that the LOD was 0.5 ng/mL blood (Dekant and Volkel 2008). That study, which examined only 19 adults, was the same one that was

unable to detect any form of BPA in urine (Volkel et al. 2005), suggesting that it is hindered by methodological problems.

Several studies that examined BPA concentrations in blood or serum from relatively healthy adults also measured BPA in individuals with diseases or health-related conditions. Two studies found women with polycystic ovarian syndrome (PCOS) had higher serum levels of BPA than did healthy control women (Takeuchi and Tsutsumi 2002; Takeuchi et al. 2004). These studies also found a positive association between serum testosterone levels and BPA concentrations; this finding is especially interesting because it provides a potential basis

Table 3. BPA levels in human serum/blood after environmental exposures (nonpregnant adults).

Reference	Detection method	LOD (ng/mL)	Sample size	Sample type	Subject description	BPA level ^a (ng/mL, mean ± SE)	Other individuals examined
Sajiki et al. 1999	Electrochemical detection or MS-ESI	0.1–0.2	12	Serum	Healthy Japanese women	Range, 0–1.6; 0.33 ± 0.54	
			9		Healthy Japanese men	Range 0.38–1.0; 0.59 ± 0.21	
Fung et al. 2000	HPLC-FD	5	40	Blood	Healthy American volunteers before dental sealant application	ND	Individuals after dental sealant application ^b
Inoue et al. 2000	HPLC with electrochemical detection	0.01 in solvent	6	Serum	Healthy Japanese adults	Mean 0.32	
	Coulometric array	0.05 in serum					
Inoue et al. 2001	LC-MS	0.1	3	Blood or plasma ^c	Healthy Japanese adults	ND–1.0	
Kezuki et al. 2002	ELISA	0.3 in serum	30	Serum	Healthy Japanese women	2.0 ± 0.146	Pregnant women, fetuses ^d
Takeuchi and Tsutsumi 2002	ELISA	0.3 in serum	11	Serum	Healthy Japanese men	1.49 ± 0.11	Women with PCOS ^e
			14	Serum	Healthy Japanese women	0.64 ± 0.1	
Yoshimura et al. 2002	GC-MS with NCI	5 pg/mL	20	Pooled serum (≥ 5 individuals per pool)	NR	0.54 ± 0.037	
Kuroda et al. 2003	HPLC fluorescence derivation, column switching	0.04	21	Serum	Sterile Japanese women	0.46 ± 0.044	Pregnant women, fetuses ^d
Hirai et al. 2004	ELISA	0.5 (from Kodaira et al. 2000)	11	Serum	Healthy Japanese women	2.5 ± 0.452	Women with endometrial hyperplasias and cancer ^f
Takeuchi et al. 2004	ELISA	0.3 in serum	19	Serum	Healthy Japanese women	0.71 ± 0.09	Women with obesity, PCOS, or both ^g
Sugiyama-Ogasawara et al. 2005	ELISA	0.5 (from Kodaira et al. 2000)	32	Serum	Healthy Japanese women	0.77 ± 0.067	Women with recurrent miscarriage ^h
Volkel et al. 2005	LC-MS/MS	0.57–1.14	19	Plasma	NR	ND	
Liu et al. 2007	LC-DAD-MS	0.05	10	Serum	Healthy Chinese adults	ND–0.28	
Dirtu et al. 2008	SPE-GC-ECNI-MS	0.003	7	Individual serum samples	Healthy Belgian adults	0.98 ± 1.09	
			14	Pooled serum samples	Healthy Belgian women	1.17 ± 1.09	
He et al. 2009	HPLC	0.39	404	Serum	Healthy Chinese men	GM, 0.20	
			482	Serum	Healthy Chinese women	GM, 0.16	
Kaddar et al. 2009	RIA	0.08	207	Plasma	French hospital patients, unknown health status	ND to > 2, detected in 83%	Patients with regular dialysis treatment ⁱ
Yang M et al. 2009	HPLC-FD	0.012	82	Blood	Healthy Korean women	Median, 0.03	Breast cancer patients ^j

Abbreviations: DAD, photodiode array detection; ECNI, electron capture negative ionization; ESI, electrospray ionization; FD, fluorometric detection; GM, geometric mean; NCI, negative chemical ionization; NR, not reported; ND, not detected; SPE, solid-phase extraction.

^aIn studies where two populations were compared, concentrations reported are for healthy controls only. ^bUnconjugated BPA was not detected after application of dental sealants. ^cThe authors interchange “blood” and “plasma,” so it is difficult to determine which was actually assessed. ^dSee Table 4. ^eMean concentration in women with PCOS, 1.04 ± 0.1 ng/mL. ^fMean concentration in women with simple endometrial hyperplasias (benign), 2.9 ± 0.632 ng/mL; women with complex endometrial hyperplasias (malignant potential), 1.4 ± 0.133 ng/mL; women with postmenopausal endometrial cancer, 1.4 ± 0.189 ng/mL. ^gMean concentration in women with obesity (no PCOS), 1.04 ± 0.09 ng/mL; PCOS (no obesity), 1.05 ± 0.10 ng/mL; obesity and PCOS, 1.07 ± 0.16 ng/mL. ^hMean concentration in women with recurrent miscarriage, 2.59 ± 0.780 ng/mL. ⁱMean concentration not provided for dialysis patients; > 70% had > 10 ng/mL. ^jMedian concentration for breast cancer patients, 0.61 ng/mL.

for gender-biased exposures or metabolism of BPA (Takeuchi et al. 2006).

Because of concerns that BPA alters the development of rodents exposed during gestation, several human biomonitoring studies have focused on measuring BPA in serum from pregnant women, and in plasma, serum, and tissue from umbilical cords (Ikezuki et al. 2002; Kuroda et al. 2003; Lee et al. 2008; Padmanabhan et al. 2008; Schönfelder et al.

2002; Tan and Ali Mohd 2003; Todaka and Mori 2002; Yamada et al. 2002) (Tables 4 and 5). Of the eight studies examining these populations, six used analytical chemistry and two used ELISA. Regardless of the method used for detection, every study was able to detect unconjugated BPA in at least some of the samples collected. Interestingly, the levels detected in pregnant women were typically higher than those reported for nonpregnant

adults; several studies that used analytical chemistry to assess BPA concentrations measured mean concentrations in the blood or serum from pregnant women at > 4 ng/mL (Lee et al. 2008; Padmanabhan et al. 2008; Schönfelder et al. 2002). However, only two studies collected samples from pregnant and nonpregnant women and directly compared them using identical methods; one reported slightly lower levels in pregnant women

Table 4. BPA levels in human serum and blood during pregnancy (maternal) or gestation (fetal).

Reference	Detection method	LOD (ng/mL)	Sample size	Sample type	Study population	BPA level [ng/mL (ppb), mean ± SE]
Ikezuki et al. 2002	ELISA	0.3 (serum)	37	Maternal serum ^a	Japanese women in early pregnancy	1.5 ± 0.197
			37	Maternal serum	Japanese women in late pregnancy	1.4 ± 0.148
			32	Fetal (cord) serum		2.2 ± 0.318
Schönfelder et al. 2002	Derivatization- GC/MS	0.01 (serum)	37	Fetal (cord) serum		2.9 ± 0.411
			37	Maternal serum	German women at delivery	4.4 ± 0.641
Yamada et al. 2002	ELISA	0.5	200	Maternal serum	Japanese women carrying fetuses with normal karyotypes	Median, 2.24
			48	Maternal serum	Japanese women carrying fetuses with abnormal karyotypes	Median, 2.97
Kuroda et al. 2003	HPLC fluorescence derivation, column switching	0.04	9	Maternal serum ^a	Japanese women at delivery	0.46 ± 0.067
			9	Fetal cord serum		0.62 ± 0.043
Tan and Mohd 2003	GC-MS	0.05	180	Fetal cord plasma	Samples collected in Malaysia	Range, ND–4.05 (88% with positive detection)
Lee et al. 2008	HPLC-GC-MS	0.625	300	Maternal blood	Korean women at delivery	9.04 ± 0.81
			300	Fetal (cord) blood		Range, ND–66.48 1.13 ± 0.08
Padmanabhan et al. 2008	HPLC-ESI-MS/MS	0.5	40	Maternal blood	American women at delivery	Range, ND–8.86 5.9 ± 0.94
						Range, ND–22.3

Abbreviations: ESI, electrospray ionization; ND, not detected.

^aData on nonpregnant females is included in Table 3.

Table 5. BPA levels in human tissues and fluids during pregnancy and lactation.

Reference	Detection method	LOD (ng/mL)	Sample size	End point(s)	Study population	BPA level [ng/mL (ppb), mean ± SE]
Ikezuki et al. 2002	ELISA	0.3	32	Early amniotic fluid (15–18 weeks)	Pregnant Japanese women	8.3 ± 1.573
			38	Late amniotic fluid (at full term, before delivery)	Pregnant Japanese women undergoing cesarian section	1.1 ± 0.162
			36	Follicular fluid	Japanese women undergoing IVF procedures	2.4 ± 0.133
Schönfelder et al. 2002	Derivatization- GC-MS	0.01	37	Placenta	German women at delivery	11.2 ± 1.512 ng/g
Todaka and Mori 2002	GC-MS	NR	NR	Umbilical cord tissue at birth	Samples collected in Japan	Mean, 4.4 ± 1.5 ng/g; range, 0.1–15.2 ng/g
Yamada et al. 2002	ELISA	0.5	200	Normal fetal amniotic fluid (14–18 weeks)	Pregnant Japanese women	Median, 0.26; range, ND–5.62
			48	Abnormal fetal karyotype fetal amniotic fluid (14–18 weeks)	Pregnant Japanese women	Median, 0
Otake et al. 2003	SPE-GC-MS	0.09	3	Breast milk	Japanese women	Range, ND–0.70 ng/g
Sun et al. 2004	DIB-Cl derivatization- HPLC	0.11	23	Breast milk	Japanese women	0.61 ± 0.042
Engel et al. 2006	HPLC electrochemical detection	0.5	21	Residual amniotic fluid from amniocentesis, < 20 weeks gestation	American women > 35 years of age	Mean, 0.55 (10% > 0.5 ng/mL)
Ye et al. 2006	Online SPE-HPLC-MS/MS	0.28	20	Breast milk	American women	Unconjugated BPA: mean, 1.3; 60% detection Total BPA: mean, 1.9; 90% detection
Kuruto-Niwa et al. 2007	ELISA	0.3	101	Human colostrum	Japanese women 3 days after delivery	3.41 ± 0.013
Ye et al. 2008a	Online SPE-HPLC-MS/MS	0.3	4	Breast milk	American women	Unconjugated BPA: mean, 0.80 Total BPA: mean, 1.02
Kaddar et al. 2009	RIA	0.08	17	Follicular fluid	French women undergoing IVF procedures	Range, ND–1.0; 39% detection

Abbreviations: DIB-Cl, 4-(4,5-diphenyl-1H-imidazol-2-yl)benzoyl chloride; ND, not detected; SPE, solid-phase extraction.

(Ikezuki et al. 2002), and the other found no differences in these two populations (Kuroda et al. 2003). Thus, this is also an important area for future research.

Of the four studies that measured unconjugated BPA in umbilical cord blood (Table 4), two detected levels higher in fetal blood than in maternal samples (Ikezuki et al. 2002; Kuroda et al. 2003), and two detected levels higher in maternal samples than in fetal blood (Lee et al. 2008; Schönfelder et al. 2002). Interestingly, two of these studies detected concentrations in a range (~ 1 ng/mL) similar to those measured in healthy adults (Kuroda et al. 2003; Lee et al. 2008). The other two studies measured BPA concentrations two to four times higher in fetuses than in nonpregnant women (Ikezuki et al. 2002; Schönfelder et al. 2002). As with the maternal blood samples, only two studies directly compared plasma/blood BPA concentrations in fetuses and nonpregnant adults (Ikezuki et al. 2002; Kuroda et al. 2003), so it is difficult to make any conclusive comparisons regarding internal concentrations, metabolism, or exposure.

With the exception of one recent study that measured BPA concentrations in the blood of 300 women and their fetuses (Lee et al. 2008), the studies mentioned above are limited because of their relatively small sample sizes. This one large-scale study (Lee et al. 2008) is the most robust blood biomonitoring study of BPA to date. Despite the relatively high LOD (0.625 ng/mL) for the method used, total BPA was detected in the blood of 84% of pregnant women and 40% of fetal samples collected; 23% of blood samples collected from pregnant women contained total BPA concentrations > 10 ng/mL, but concentrations of unconjugated BPA alone were not measured.

The overall consensus that can be determined from blood sampling of healthy adults, adults with certain diseases, pregnant women, and fetuses is that internal exposures to unconjugated BPA are in the range of 0.5–10 ng/mL, with most studies suggesting an average internal exposure of approximately 1–3 ng/mL (Vandenberg et al. 2007). These concentrations are higher than those required to stimulate responses in cell cultures (Wetherill et al. 2007), suggesting that these low levels could influence biological end points and development in humans (vom Saal et al. 2007). At this time, no information is available on BPA concentrations in the blood of infants after birth or of children and adolescents, indicating a significant data gap. Because neonates are not thought to have the metabolic enzymes to effectively conjugate BPA (Mykkanen et al. 1997; Taylor et al. 2008), it is plausible and even likely that these individuals have higher concentrations of unconjugated BPA in their blood than do adults (de Wildt et al. 1999).

BPA Measurements in Amniotic Fluid, Placenta, and Follicular Fluid

Only a few studies have examined additional tissues and fluids associated with pregnancy (Table 5). In 2002, Schönfelder et al. (2002) measured unconjugated BPA concentrations in placental tissue from 37 pregnancies. BPA was detected in all samples, ranging from 1.0 to 104.9 ng/g tissue, with a median value of 12.7 ng/g and a mean value of 11.2 ng/g. This study suggests that BPA is transplacentally transferred to the embryo/fetal compartment. Additional studies support the idea that the fetus is continuously exposed to BPA; three studies have demonstrated that it can be measured in amniotic fluid (Engel et al. 2006; Ikezuki et al. 2002; Yamada et al. 2002). Ikezuki et al. (2002) found eight times higher concentrations of unconjugated BPA in amniotic fluid from early pregnancy compared with later pregnancy; they proposed that BPA may accumulate in early fetuses because of a lower metabolic clearance of BPA or may be conjugated more efficiently in the fetal liver during later gestation. In another study, Engel et al. (2006) detected unconjugated BPA in $< 10\%$ of the amniotic fluid samples collected from pregnancies before 20 weeks of gestation. Collectively, these studies lend support to the idea that the fetus is exposed to unconjugated BPA. In summary, additional studies using larger sample sizes, collection of samples controlled for BPA contamination, and sensitive methods of detection are needed to more accurately quantify BPA in amniotic fluid and placental tissue. However, published studies indicate that the fetus is likely exposed to unconjugated BPA via maternal uptake.

With an increase in the number of successful pregnancies that have resulted from assisted reproductive technologies, two studies examining unconjugated BPA levels in follicular fluid raise additional concerns (Ikezuki et al. 2002; Kaddar et al. 2009) (Table 5). First, Ikezuki et al. (2002) found an average concentration of 2.4 ng/mL fluid collected from women undergoing *in vitro* fertilization (IVF) procedures. Second, Kaddar et al. (2009) measured BPA in follicular fluid from 28 infertile women undergoing IVF. The authors detected unconjugated BPA in 11 of these samples (39%) at concentrations that ranged from 0.15 to 1 ng/mL fluid. These studies have several limitations, including the analytical methods used and the selection of human subjects. However, studies from rodents suggest that BPA can cause aneuploidy in oocytes (Susiarjo et al. 2007) and alter the body weight of offspring resulting from intrauterine transplantation of embryos cultured in media containing BPA (Takai et al. 2001). As such, presence of measurable

concentrations of unconjugated BPA in follicular fluid is a potential concern.

BPA Exposure Assessment in Breast Milk

There is significant interest in defining all sources of BPA exposure, especially those specific to neonates, infants, and children; baby bottles and packaging of infant formulas are currently identified sources of oral BPA exposure (Gies et al. 2009; Vandenberg et al. 2007). An additional and important consideration for the health of the developing neonate is potential BPA exposure from breast milk. BPA is somewhat lipophilic [K_{ow} (octanol–water partitioning coefficient) = 2.2–3.4], allowing it to partition into fat and breast milk. Four small studies using analytical chemistry have measured BPA in the breast milk of healthy women (Table 5). The first study examined breast milk from three women and found detectable levels of unconjugated BPA in two of the samples (Otaka et al. 2003). In another study, Sun et al. (2004) detected unconjugated BPA (range, 0.28–0.97 ng/mL; mean, 0.61 ng/mL) in the breast milk of 100% of the women studied. A third study detected unconjugated BPA in 60% of samples (mean, 1.3 ng/mL milk) and total BPA in 90% (mean, 1.1 ng/mL milk) (Ye et al. 2006). In the fourth study, Ye et al. (2008a) detected unconjugated and total BPA in all four of the samples examined.

In a larger study, Kuruto-Niwa et al. (2007) examined BPA concentrations in human colostrum, the milk produced within the first 3 days after giving birth that contains high levels of antibodies, carbohydrates, and protein. Using ELISA, the authors reported detecting unconjugated BPA in 100% of the 101 samples examined (range, 1–7 ng/mL colostrum; mean, 3.41 ng/mL). Although the sample sizes in these studies are relatively small, these findings highlight the concerns about exposure of human infants to BPA via breast milk in addition to other exposures from baby bottles and other containers.

BPA Exposure Assessment in Adipose Tissue

To address the potential for BPA to accumulate in adipose tissue, Fernandez et al. (2007) examined 20 adult women from Spain for both unconjugated BPA and chlorinated BPA derivatives. Chlorinated BPA derivatives are thought to form when BPA is conjugated in treated water sources. Fernandez et al. (2007) detected unconjugated BPA in 55% of the samples and Cl_2BPA in 80% of samples; other chlorinated derivatives, including ClBPA and Cl_3BPA , were detected less frequently, and Cl_4BPA was not detected in any sample. Of those samples in which BPA was detected, concentrations ranged from 1.80 to

12.01 ng/g adipose tissue (mean, 3.16 ng/g) (Fernandez et al. 2007). In another study, Nunez et al. (2001) reported that repeated exposure to relatively high BPA doses resulted in the distribution of BPA to bodily tissues in rats, including adipose tissue. Human biomonitoring studies using adipose tissue are understandably quite limited because of the invasive procedures needed to isolate this biological matrix, so it is not likely that this study will be repeated with a larger sample size or a broader population. However, it does point out that there are other metabolites of BPA that should be examined for biological and hormonal activity.

Relationships between Measured BPA Concentrations and Disease Outcomes

In 2008, a large and well-controlled study of the possible health effects of BPA exposure on humans was conducted using samples and information collected for NHANES (Lang et al. 2008). By examining data from 1,455 American adults, these authors found positive associations between urinary (total) BPA concentrations and the prevalence of diabetes, heart disease, and liver toxicity. In a recent study, Melzer et al. (2010) retested the originally identified associations between higher urinary total BPA concentrations and reported heart disease using a second NHANES database. Indeed, the authors were able to replicate earlier associations that were detected between higher urinary concentrations of total BPA and an increased prevalence of coronary heart disease, even though urinary BPA concentrations in this second study were substantially lower than those measured in the first (Lang et al. 2008). In both of these studies, adult exposure levels were associated with chronic diseases that probably began much earlier in the individual's life. Also of note is that, as with most epidemiological studies, it is impossible to determine cause-and-effect relationships between measured concentrations and disease states. Therefore, the authors cannot determine whether higher concentrations of BPA cause these conditions, or if the presence of these diseases leads to increased exposure or decreased metabolism of BPA; either of these situations is potentially concerning. Additional studies are therefore needed to determine whether there is a causal link between elevated BPA concentrations in urine and these chronic diseases. In addition, it should be noted that these studies used only single urine collections from the individuals examined (Lang et al. 2008; Melzer et al. 2010); multiple collections from individuals over a range of life stages, especially encompassing periods of organogenesis, would be more useful for determining causative relationships.

Another recent study examined BPA concentrations in urine and blood collected from 516 Korean adults living in urban areas (Hong et al. 2009). Urinary concentrations of total BPA were associated with at least one marker of oxidative stress, although these associations were no longer statistically significant when age, sex, weight, smoking, and exercise were considered in the regression models. Hong et al. (2009) also detected an association between high BPA exposures and increased fasting blood glucose levels, perhaps lending additional weight to a possible link between BPA exposure and diabetes. Another study supports the connection between BPA exposure and oxidative stress; Yang YJ et al. (2009) found that total BPA levels were associated with markers of stress in postmenopausal women, but not in premenopausal women or men, leading the authors to suggest that postmenopausal women may be more susceptible to BPA-induced adverse health effects.

Several smaller studies have examined the effects of BPA exposure on other health outcomes. For instance, BPA levels in blood have been associated with a variety of conditions in women, including obesity, endometrial hyperplasia, endometriosis, recurrent miscarriages, sterility, and PCOS (reviewed by Vandenberg et al. 2007). Other studies detected associations between high BPA exposure and chromosomal abnormalities, including pregnancies with fetuses that had an abnormal karyotype (Yamada et al. 2002), recurrent miscarriage (Sugiura-Ogasawara et al. 2005), and sister chromatid exchange measured in peripheral lymphocytes (Yang et al. 2006). These epidemiological studies have several limitations, including small sample sizes, insufficient information on subject selection criteria, and cross-sectional designs that failed to adequately control for potential confounders (Vandenberg et al. 2007), thus preventing accurate assessments regarding the potential health risks of BPA. Even more recent studies have identified relationships between BPA exposure and reproductive hormone levels in male patients at an infertility clinic (Meeker et al. 2010) and the number of oocytes retrieved from women undergoing IVF fertility treatments (Mok-Lin et al. 2009). Finally, prenatal BPA exposures, as determined by maternal urine concentration during pregnancy and at delivery, were found to be associated with increased externalizing behaviors (i.e., aggression, hyperactivity), especially in female toddlers (Braun et al. 2009).

It is somewhat surprising that most studies undertaken to understand human health hazards have relied heavily on biomonitoring of BPA from the general population, and only a few studies have measured exposures to BPA in occupational settings or have associated occupational exposures with adverse health

outcomes. This limitation may stem from the belief that BPA exposure comes predominantly from contamination via food sources. Despite reports suggesting exposure of healthy individuals to BPA by non-oral routes (Stahlhut et al. 2009), a data gap exists relative to exposure levels in employees in BPA-producing industries or those workers using BPA in different manufacturing processes. Thus, measurement strategies and studies to investigate occupational exposure to BPA and health outcomes are very much needed.

Reliability of Biomonitoring and Toxicokinetic Studies

The need for human biomonitoring of BPA for risk assessment purposes is undisputed because all sources of exposure have not been identified, and thus internal exposures cannot be properly calculated. Groups charged with assessing risk from exposure have heated debates about the toxicokinetics of BPA and the plausibility and reliability of individual data and analytical approaches used in biomonitoring studies. Two studies thus far have attempted to determine the kinetics of BPA metabolism in human subjects (Volkel et al. 2002, 2005).

The first toxicokinetic study was designed to resolve issues regarding the internal exposure to BPA that originated from animal studies: Volkel et al. (2002) administered 5 mg deuterium-labeled BPA (equivalent to 54–90 µg/kg BW) orally to adult volunteers and monitored blood and urinary BPA levels. Unconjugated BPA was always < LODs in both plasma (LOD = 2.28 ng/mL) and urine (LOD = 1.37 ng/mL) at all time points studied after BPA administration; the results were interpreted by the authors as indicative of rapid metabolism, noting that “only a small percentage of the dose of [BPA] is available for other biotransformation pathways, due to the rapid glucuronidation” (Volkel et al. 2002). In support of this statement, concentrations of conjugated BPA were also measured in blood and urine; the authors reported that BPA glucuronide concentrations fell below the LOD in both urine and blood 24–34 hr after BPA administration, although this is not apparent from the concentration–time course model they presented. Using their finding that the terminal half-life of BPA glucuronide in blood was 5.3 hr, Volkel et al. (2002) concluded that conjugated BPA is also rapidly cleared from the blood.

Several inconsistencies in the article by Volkel et al. (2002) raise questions about its reliability. For instance, the authors reported two different values for the time when maximal plasma concentrations (C_{max}) were achieved (1.35 hr vs. 4 hr). In addition, the BPA glucuronide levels reported in blood are higher than the total BPA concentrations

measured in the same individuals. Finally, the authors indicated that they measured BPA metabolism in three women, a group of three men, and then in a separate group of four men, yet the groups of male volunteers clearly overlap (at least 2 men were subjects in both groups), making the data compiled from combining these two groups questionable.

In drawing their conclusion that there is no risk from current human exposure levels, Volkel et al. (2002) overlooked several important points from a risk assessment perspective. First, they did not acknowledge the likelihood of different toxicokinetics when BPA exposure is continuous compared with a single administration. Ginsberg and Rice (2009) suggested that results from the Volkel et al. (2002) study were more consistent with delayed excretion from long-term internal storage or cycling between conjugation and deconjugation. Second, the potential for BPA to have actions at low levels (in the nanograms per milliliter range) was not considered. Third, this toxicokinetic study was designed to assess the metabolism of BPA after oral exposure because, until very recently (Stahlhut et al. 2009), it was assumed that most if not all BPA exposure in humans occurs via the oral route. However, because all sources of BPA have not been identified, non-oral exposures cannot be discounted. Finally, Volkel et al. (2002) overlooked the possibility of differences in toxicokinetics under different physiological paradigms; they used a small mixed-subject group composed of individuals of both sexes and different ages. The differences between sexes and age groups in urinary BPA levels found in CDC biomonitoring studies (Calafat et al. 2005, 2008) raise the possibility that the toxicokinetics of chemicals and drugs, including BPA, are likely to be very different in fetuses and neonates compared with adults. In fact, this assumption was confirmed in two independent kinetic models, which found that the internal exposure to BPA in newborns can be 3–10 times higher than in adults (Edginton and Ritter 2009; Mielke and Gundert-Remy 2009). There is also the potential for deconjugation of BPA glucuronide *in utero* by β -glucuronidase, an enzyme that is present in high concentrations in placenta and various other tissues (Ginsberg and Rice 2009). Furthermore, studies in rodents have found that neonates have limited ability to convert BPA into an inactive conjugated form, independent of the route of administration (Taylor et al. 2008). This may also be true for human fetuses and neonates.

In a follow-up investigation, Volkel et al. (2005) further examined the kinetics of BPA in urine and plasma. They assessed BPA exposure both after administration of labeled BPA and in an environmentally exposed population. For direct testing, subjects were

administered 25 μ g labeled BPA (equivalent to 0.28–0.43 μ g/kg BW), a much smaller dose than that administered in their earlier study; unconjugated and conjugated BPA were then measured in urine (Volkel et al. 2005). Although the authors suggested that they had developed a very sensitive and selective method, the LODs for this study were again higher than those in other studies using the same analytical methods (for unconjugated BPA, LOD = 1.14 ng/mL; for BPA glucuronide, LOD = 10.1 ng/mL).

Volkel et al. (2005) examined BPA kinetics in six individuals administered BPA, although they provided no information about the characteristics of these subjects, making it difficult to draw any conclusions from their study. The authors suggested that there were no differences in kinetics among volunteers, yet a closer look at their results shows a wide variation in BPA measurements across individuals. In the three men examined, 85% of the administered BPA dose was recovered in urine after 5 hr, mostly as BPA glucuronide. In the three women examined, 75% of BPA was recovered as BPA glucuronide after the same period of time. In two of six individuals, unconjugated BPA was detected at levels of approximately 1 ng/mL urine. This finding directly contradicts the conclusions reached by the study authors, who suggested that 100% first-pass metabolism would promptly convert BPA to its conjugated metabolites. As mentioned above, several other studies have also detected unconjugated BPA in urine (Calafat et al. 2009; Kim et al. 2003; Ouchi and Watanabe 2002; Schöringhumer and Cichna-Markl 2007; Volkel et al. 2008; Ye et al. 2005b). Volkel et al. (2005) also collected plasma samples for measurement of BPA, although no measurements of conjugated or unconjugated BPA were reported. Finally, unlike other biomonitoring studies, these investigators failed to detect any BPA in the environmentally exposed group, likely because the LODs in these kinetic studies were 10 to 100 times less sensitive than methods used in most biomonitoring studies. These limited observations emphasize the need for risk assessment studies to employ state-of-the-art analytical techniques that are sensitive enough to detect low levels of BPA with large sample sizes so that one can consider variability in the population and influence from physiological status; these kinetic studies therefore are not appropriate for risk assessment of BPA.

The next issue to consider is whether biomonitoring studies *per se* are sufficiently reliable and therefore useful for risk assessment purposes. The detection rates and concentrations of BPA in urine and blood of environmentally exposed individuals are remarkably similar in studies performed by

several independent groups using state-of-the-art analytical techniques; independent confirmation of these results alone indicates that these studies are reliable. These findings are further supported by the dozens of additional studies that have used less sensitive or less selective methodologies, because all of these studies report conjugated or unconjugated BPA levels in human samples in a very narrow range of concentrations. Taken together, although the discussions about the reliability of unconjugated BPA measurements within human tissues and fluids appear to be ongoing, especially in the risk assessment community, the consistency of available data on internal exposure to BPA across investigative groups is more than convincing.

Several biomonitoring studies (Calafat et al. 2005, 2008; Kim et al. 2003; Mao et al. 2004) and one of the toxicokinetic studies (Volkel et al. 2005) suggest that men and women differ in their uptake and/or metabolism of BPA. Other studies suggest that pregnant and nonpregnant women differ in their uptake or metabolism of chemicals, including BPA. These findings reinforce the fact that kinetic models based on metabolism of BPA from a mixed group of adults cannot be extrapolated to different physiological states, especially vulnerable populations such as pregnant women and children.

Utility of Physiologically Based Toxicokinetic Models of BPA

Physiologically based toxicokinetic (PBTK) modeling incorporates information on the physiology and anatomy of the experimental animal or human and the biochemistry of the chemical of interest into a conceptual model for computer simulation. Compared with the essentially empiric kinetic models (i.e., the so-called classical kinetic models), PBTK modeling has the advantage of being more grounded in physiology. PBTK models are powerful tools for interpolations and extrapolations that are particularly useful in the context of risk assessment, such as dose to dose, route to route, single to multiple exposure, continuous to discontinuous exposure, species to species, "external" or administered dose and internal or target tissue concentration, males to females, adults to children, nonpregnant women to pregnant, and so on (Loizou et al. 2008).

A few PBTK models have been developed for describing the toxicokinetics of BPA in rats (Shin et al. 2004), pregnant mice (Kawamoto et al. 2007), adult humans (Teeguarden et al. 2005), and children < 2 years of age (Edginton and Ritter 2009). First, Shin et al. (2004) developed a PBTK model involving vein, artery, lung, liver, spleen, kidneys, heart, testes, muscle, brain, adipose tissue, and small intestines for predicting tissue distribution and kinetics of BPA in rats. The model was

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validated by predicting the steady-state levels of BPA in the blood and tissues of rats that had received repeated intravenous injections. These investigators used their PBTK model to simulate blood and tissue levels of unconjugated BPA in an adult human (70 kg BW) receiving multiple oral doses of BPA (100 mg every 24 hr for 10 days) and found steady-state venous blood levels of 1.3 ng/mL, a concentration remarkably similar to what is measured in environmentally exposed humans (Table 3).

In a model of oral route exposure to BPA in rats and humans, Teeguarden et al. (2005) imposed restrictions on the concentration of unbound (free) BPA resulting from plasma protein binding and predicted the degree of ER binding that may occur in the rat uterus at different BPA doses. Based on simulations using the model, terminal BPA elimination in rats, but not humans, was found to be strongly influenced by the enterohepatic recirculation of BPA glucuronide. The oral route blood kinetics in rats and the oral route plasma and urinary elimination kinetics in humans described by the model of Teeguarden et al. (2005) were consistent with data reported by Pottenger et al. (2000) and Volkel et al. (2002) for BPA-treated rats and human volunteers, respectively. However, their model was based on single exposures to BPA and did not account for repeated exposures to humans, as actually occurs. Nonetheless, as far as toxicokinetic modeling in humans is concerned, plasma levels of unconjugated BPA predicted by the physiologically based pharmacokinetic (PBTK) model for a single oral dose of 5 mg BPA per person were less than the analytical LOD of the method used by Volkel et al. (2002). Thus, only appropriately sensitive analytical methods should be used to determine metabolic parameters after administration of BPA.

More recently, a PBTK model was developed to assess the age dependence of the toxicokinetics of both unconjugated BPA and BPA glucuronide in children < 2 years of age (Edginton and Ritter 2009). This PBTK model was initially built using information gathered from toxicokinetic studies of BPA in adults and then scaled down taking into account the age dependence of physiological parameters relevant for absorption, distribution, metabolism, and elimination. According to the model predictions, steady-state plasma concentrations of unconjugated BPA in newborns were expected to be 11 times higher than those found in adults who received the same body weight-normalized dose. Taking into account estimates of dietary exposures, plasma levels of unconjugated BPA in 3- to 6-month-old children were expected to be 5 times higher than the levels found in adults. Simulations using this PBTK model

are therefore consistent with the idea that newborns and young children are internally exposed to higher levels of unconjugated BPA than levels that have been estimated for adults. Indeed, data from infants in neonatal infant care units in American hospitals suggested that BPA levels in these infants were 11 times higher than GM concentrations in American adults (Calafat et al. 2009), matching exactly the predictions from the PBTK model.

Another PBTK model—one for BPA in pregnant mice—was developed by Kawamoto et al. (2007). The authors used experimental kinetic data from a single oral dose of BPA administered to pregnant mice on gestational day 15 for calibrating their kinetic model. Simulations using their PBTK model indicated that (total) BPA was rapidly transferred through the placenta to the fetus and that it was only slowly eliminated from the fetal compartment. It should be stressed that this model was calibrated using data for total (unconjugated plus conjugated) BPA, so it did not specifically estimate the toxicokinetics of unconjugated BPA. As far as we are aware, no other PBTK model for BPA in pregnant rodents or pregnant humans has been constructed. Additional and more refined PBTK models of unconjugated BPA in pregnant rodents and humans are therefore urgently needed.

Current PBTK models for BPA do not take into consideration the influence of local deconjugation of BPA metabolites at or in the vicinity of target tissues. Ginsberg and Rice (2009) recently discussed literature reports supporting the biological plausibility of local deglucuronidation of BPA glucuronide occurring at a number of target tissues, such as placenta and the fetal compartment. For instance, Takahashi and Oishi (2000) demonstrated that placenta has rather extensive β -glucuronidase activity and that liver and kidneys show much higher unconjugated BPA levels than blood. More information is needed to determine if, in fact, BPA is deconjugated *in situ*; if so, this should be added to future models.

Finally, a recent study determined that there is a linear relationship between applied doses and circulating unconjugated BPA concentrations (Vandenberg et al. 2007). This linear relationship was used to back-calculate the applied dose needed to generate the concentrations of unconjugated BPA that have been repeatedly measured in environmentally exposed humans; the applied dose needed to achieve circulating BPA levels of 1–3 ng/mL blood that have been detected in biomonitoring studies (Table 3) exceeded 500 μ g/kg/day. This matches the predictions from the PBTK model developed by Shin et al. (2004), which clearly indicated that doses of 100 mg every 24 hr for 10 days were needed to produce blood levels of 1.3 ng/mL in rats. These data

need to be verified, but if true, they provide strong evidence that one cannot use urine levels to back-calculate to exposure levels.

Future Directions and Research Needs

Throughout this review we have mentioned research needs; these are summarized below.

We suggest that several small biomonitoring studies performed previously should be repeated using large reference populations. First, it is imperative that estimates of urinary BPA levels over 24 hr be undertaken. Second, there is a need for obtaining multiple samples of blood and urine from individuals to assess variability of exposure over time, likely encompassing weeks, months, or years. Finally, large-scale biomonitoring studies across the life span are needed to confirm levels of total and unconjugated BPA in blood. These studies need to compare exposure levels in men and women, adults, neonates, children (spanning the prepubertal to pubertal period), pregnant and nonpregnant women, and obese and nonobese individuals, and across disease states. It is clear from animal studies that developmental periods (*in utero* and neonatal periods) are the most sensitive to BPA; thus, special attention should be given to assessment of total and unconjugated BPA in these vulnerable populations.

More studies are needed to examine pregnancy outcomes from large groups of racially and socioeconomically diverse women, relating them to internal exposure levels throughout pregnancy. It has been hypothesized that racial disparities in exposures to BPA and other endocrine-disrupting chemicals may account for the reduced fetal survival and birth weight of offspring (Ranjit et al. 2010). Recent epidemiology studies suggest relationships between urinary BPA concentrations and fertility end points (Meeker et al. 2010; Mok-Lin et al. 2009). Additional studies are needed to extend these findings and also to define the levels of total and unconjugated BPA in ovarian follicular fluid, semen, amniotic fluid, and cord blood to relate to reproductive disease outcomes and adult consequences.

Human toxicokinetic studies of BPA that employ sensitive methods across physiological states and age groups are needed. In addition, studies directly comparing the toxicokinetics of BPA metabolism in humans and laboratory animals will help to determine if animals can accurately predict human metabolism.

Studies are needed to identify all sources of exposure and to assess the daily dose of BPA actually coming from the oral route versus other routes. Measurement of BPA in all food and drink sources and in indoor and outdoor air samples could be mandated by legislative actions.

Longitudinal studies in children linking developmental exposures to BPA and later-onset diseases are especially important and needed. Two epidemiological studies have related adult urinary BPA concentrations to disease outcomes such as obesity, type 2 diabetes, and cardiovascular disease (Lang et al. 2008; Melzer et al. 2010), all of which are on the rise. Studies that focus on relating BPA exposure to disease outcomes should also control for dietary phytoestrogen exposure and consider mixture effects.

In addition to these specific data needs, it is important to take advantage of the NHANES data and database (<http://www.cdc.gov/nchs/nhanes.htm>) for assessment of BPA levels and the relationship of those levels and disease outcomes. Several recent studies are excellent examples of using this database to answer important questions relating to BPA exposure and linking BPA exposures to disease outcomes (Lang et al. 2008; Stahlhut et al. 2009). The NHANES database is a virtual treasure of data awaiting analysis and comparison.

Conclusions

We believe that human biomonitoring data clearly indicate that the general population is exposed to BPA ubiquitously, including significant internal exposures to unconjugated BPA. More important, animal studies suggest that fetuses and children are particularly vulnerable to BPA exposures and, at the same time, are exposed to higher levels of unconjugated BPA.

The two toxicokinetic studies performed to date (Volkel et al. 2002, 2005), which suggest that human exposure is negligible, have significant flaws and are therefore not reliable for risk assessment purposes. Further, the biomonitoring data, coupled with predictions from PBTK models, indicate that human exposures are higher than have been suggested from the toxicokinetic studies. Weighing all the evidence available to date—because of the significant data showing human exposures to unconjugated BPA and animal data indicating increased susceptibility to disease at levels found in humans—we recommend that the precautionary principle be followed until further data are available on exposure of fetuses and children to BPA. The health of the public is at stake.

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Why Public Health Agencies Cannot Depend on Good Laboratory Practices as a Criterion for Selecting Data: The Case of Bisphenol A

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BACKGROUND: In their safety evaluations of bisphenol A (BPA), the U.S. Food and Drug Administration (FDA) and a counterpart in Europe, the European Food Safety Authority (EFSA), have given special prominence to two industry-funded studies that adhered to standards defined by Good Laboratory Practices (GLP). These same agencies have given much less weight in risk assessments to a large number of independently replicated non-GLP studies conducted with government funding by the leading experts in various fields of science from around the world.

OBJECTIVES: We reviewed differences between industry-funded GLP studies of BPA conducted by commercial laboratories for regulatory purposes and non-GLP studies conducted in academic and government laboratories to identify hazards and molecular mechanisms mediating adverse effects. We examined the methods and results in the GLP studies that were pivotal in the draft decision of the U.S. FDA declaring BPA safe in relation to findings from studies that were competitive for U.S. National Institutes of Health (NIH) funding, peer-reviewed for publication in leading journals, subject to independent replication, but rejected by the U.S. FDA for regulatory purposes.

DISCUSSION: Although the U.S. FDA and EFSA have deemed two industry-funded GLP studies of BPA to be superior to hundreds of studies funded by the U.S. NIH and NIH counterparts in other countries, the GLP studies on which the agencies based their decisions have serious conceptual and methodologic flaws. In addition, the U.S. FDA and EFSA have mistakenly assumed that GLP yields valid and reliable scientific findings (i.e., "good science"). Their rationale for favoring GLP studies over hundreds of publically funded studies ignores the central factor in determining the reliability and validity of scientific findings, namely, independent replication, and use of the most appropriate and sensitive state-of-the-art assays, neither of which is an expectation of industry-funded GLP research.

CONCLUSIONS: Public health decisions should be based on studies using appropriate protocols with appropriate controls and the most sensitive assays, not GLP. Relevant NIH-funded research using state-of-the-art techniques should play a prominent role in safety evaluations of chemicals.

KEY WORDS: bisphenol A, endocrine disruptors, FDA, Food and Drug Administration, GLP, good laboratory practices, low-dose, nonmonotonic, positive control. *Environ Health Perspect* 117:309–315 (2009). doi:10.1289/ehp.0800173 available via <http://dx.doi.org/> [Online 22 October 2008]

Regulatory agencies in the United States and the European Union (EU) have justified the decision to declare the estrogenic chemical bisphenol A (BPA) safe at current levels of human exposure based on a few studies conducted using Good Laboratory

Practices (GLP). In contrast, these agencies have rejected for consideration in their risk assessment of BPA hundreds of laboratory animal and mechanistic cell culture studies conducted by academic and government scientists reporting harm at very low doses of

BPA. These studies were rejected primarily because they were not conducted using GLP. We suggest that decisions based on this logic are misguided and will result in continued risk to public health from exposure to BPA, as well as other manmade chemicals.

GLP is a federal rule for conducting research on the health effects or safety testing of drugs or chemicals submitted by private research companies for regulatory purposes. The GLP outlines basic guidelines for conducting scientific research, including the care and feeding of laboratory animals, standards for facility maintenance, calibration and care of equipment, personnel requirements, inspections, study protocols, and collection and storage of raw data (Goldman 1988). These regulations were developed in response to widespread misconduct by private research companies; this misconduct was possible because their data usually do not go through the rigorous, multistage scientific review that is normal for academic data funded by federal agencies and published in the peer-reviewed literature. The lack of these safeguards from academic science had enabled fraud. The U.S. Food and Drug

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Administration (U.S. FDA) first issued rules for GLP in 1978 after a 2-year federal investigation into sloppy laboratory practices of a number of private research companies (Lublin 1978; Markowitz and Rosner 2002). What began as serious concerns about poor quality research expanded into a criminal investigation of Industrial Bio-Test (IBT), one of the largest private laboratories at the time and a subsidiary of Nalco Chemical Company. In response to the federal investigation, the U.S. Environmental Protection Agency (EPA) demanded that 235 chemical companies re-examine the > 4,000 tests conducted by the laboratory. In 1983, three men from IBT were found guilty of deliberating doctoring data and were sentenced to prison (Lublin 1978; Markowitz and Rosner 2002). The fraudulent practices of IBT brought into question 15% of the pesticides approved for use in the United States. That same year, the U.S. EPA issued similar GLP rules for regulatory testing.

Both the U.S. FDA (2008a) and European Food Safety Authority (EFSA 2006) have recently published documents demonstrating that their decision to continue to declare BPA safe at current exposure levels was based primarily on the results of a few industry-funded studies that followed GLP guidelines. These decisions stand in stark contrast to the decisions concerning the potential risks to human health reached by a panel of 38 experts at a U.S. National Institutes of Health (NIH)-sponsored conference, who published The Chapel Hill Consensus Statement (vom Saal et al. 2007), as well as five review articles (Crain et al. 2007; Keri et al. 2007; Richter et al. 2007a; Vandenberg et al. 2007a; Wetherill et al. 2007). These peer-reviewed articles covered approximately 700 articles concerning BPA and represented a comprehensive review of the literature as of the end of 2006. In addition, the U.S. FDA draft decision contradicted the conclusions reached by the National Toxicology Program (NTP), which had spent 2 years investigating this question (NTP 2008). An important role of the NTP is to advise the U.S. FDA about the science relating to toxic chemicals in food, but in an unusual move, the U.S. FDA chose to release its draft report before the release of the final report on BPA by the NTP and without indicating who at the U.S. FDA was involved in preparing the draft report (U.S. FDA 2008b). At a hearing on 16 September 2008 regarding the draft report on BPA, the U.S. FDA announced that their goal was to have a subcommittee of the U.S. FDA Science Board complete a review of the draft decision by the end of October 2008. This would presumably also involve review by the subcommittee members of the approximately 1,000 articles relating to BPA.

We believe that the methods employed in chemical industry-sponsored GLP studies are

incapable of detecting low-dose endocrine-disrupting effects of BPA and other hormonally active chemicals. Detecting endocrine-disrupting effects at low doses of chemicals such as BPA requires sophisticated and modern assays and analyses that have been developed in advanced, usually federally funded laboratories over the past decade. This is especially apparent when one examines what is now known about functional effects of BPA on a wide range of end points (Richter et al. 2007a; Welshons et al. 2006; Wetherill et al. 2007). These end points include those mediated by recently discovered estrogen response pathways initiated in human and animal cell membranes (nonclassical or alternative estrogen response mechanisms), which multiple laboratories have shown to be equally sensitive to BPA and estradiol in terms of activating effects in human and animal cells at low picomolar through low nanomolar concentrations (Alonso-Magdalena et al. 2008; Wetherill et al. 2007; Wozniak et al. 2005; Zsarnovszky et al. 2005).

The effects of BPA documented in these studies include a diverse array for which there are no data from GLP studies because the end points have not been examined: altered metabolism related to metabolic syndrome (Alonso-Magdalena et al. 2005, 2006, 2008; Ropero et al. 2008); altered adiponectin secretion (Hugo et al. 2008), which is a condition predicting heart disease and type 2 diabetes (Lang et al. 2008); altered epigenetic programming leading to precancerous lesions of the prostate (Ho et al. 2006); differential growth patterns in the developing prostate (Timms et al. 2005); abnormal growth, gene expression, and precancerous lesions of the mammary glands (Soto et al. 2008); and adverse effects on the female reproductive system, including uterine fibroids, paraovarian cysts, and chromosomal abnormalities in oocytes (Newbold et al. 2007; Susiarjo et al. 2007). There is also a large literature on neuroanatomic, neurochemical, and behavioral abnormalities caused by low doses of BPA (Leranth et al. 2008; Richter et al. 2007a), which also are not capable of being detected by current GLP studies conducted for regulatory purposes because of their out-of-date assays.

The approaches used by academic and government scientists to study the potential health hazards of BPA contrast sharply with those still used by the chemical industry that are relied on by regulatory agencies in the United States and Europe, including the two studies identified by both the U.S. FDA and European Food Safety Authority (EFSA) as central to the decision to declare BPA safe at current human exposure levels (Tyl et al. 2002, 2008a). By using outdated and insensitively assays that were supposed to have been

replaced by a new battery of screens and tests by 2000 [as mandated by the U.S. Congress in 1996 in the Food Quality Protection Act (1996), but which has, as yet, still not occurred], these studies conducted using GLP fail to find any adverse effects.

Reliability and Validity

Reliability and validity are separate issues, although in the experimental research described here, validity and reliability basically refer to research that is credible. Golafshani (2003) noted that "reliability" refers to the extent to which results are consistent over time and are an accurate representation of the total population under study. Of central importance is that the results of a study must be reproduced under a similar methodology to be considered to be reliable. "Validity" refers to whether the research measures what it was intended to measure, and valid findings are considered to be true. In other words, reliability is determined by whether the results are replicable, whereas validity is assessed by whether the methods used result in finding the truth as a result of the investigator actually measuring what the study intended to measure.

Use of GLP in Regulatory Decision Making

Despite strong evidence of aberrations caused by low doses of BPA in animals exposed during fetal and neonatal life in studies conducted by the world's leading academic and government experts in the fields of endocrine disruption, endocrinology, neurobiology, reproductive biology, genetics, and metabolism, a relatively small number of studies reporting no adverse effects at low doses of BPA have continued to be promoted by the chemical industry and used by regulatory agencies (e.g., Ashby et al. 1999; Cagen et al. 1999; Tyl et al. 2002, 2008a). According to the U.S. FDA, these are accepted because they used GLP (U.S. EPA 2008), with the implication that studies not employing GLP are not reliable or valid (U.S. FDA 2008a).

GLP does not guarantee reliability or validity of scientific results. Unfortunately, although GLP creates the semblance of reliable and valid science, it actually offers no such guarantee. GLP specifies nothing about the quality of the research design, the skills of the technicians, the sensitivity of the assays, or whether the methods employed are current or out-of-date. (All of the above are central issues in the review of a grant proposal by an NIH panel.) GLP simply indicates that the laboratory technicians/scientists performing experiments follow highly detailed U.S. EPA requirements [or in the EU, Organization for Economic Co-operation and Development (OECD) requirements] for record keeping, including details of the conduct of the

experiment and archiving relevant biological and chemical materials (U.S. EPA 2008).

These record-keeping procedures in GLP were instituted because of widespread misconduct being committed by commercial testing laboratories (described above). These fraudulent results were possible because contract laboratory studies used in the regulatory process are rarely subject to the checks and balances that peer-reviewed, replicated scientific findings undergo. Without that acid test of reliability (replication by other independent scientists), other procedures were needed. Hence GLP was implemented, despite its severe limitations.

NIH-funded research subject to more stringent reviews than GLP. Although few NIH-funded investigators adhere to GLP-mandated record keeping, the procedures of GLP are actually surpassed by the procedures required for NIH-funded science published in peer-reviewed journals. NIH-funded studies pass through three phases of peer review that are far more challenging than GLP requirements. First, the principal scientists must have demonstrated competence to conduct the research, and experimental methods, assays, and laboratory environment must involve use of state-of-the-art techniques to be competitive for NIH funding. Second, results are published in peer-reviewed journals, with detailed evaluations by independent experts examining all aspects of the study. And third, the findings are challenged by independent efforts to replicate; for example, the initial findings concerning the stimulating effects of estrogenic chemicals on the mouse prostate (Nagel et al. 1997; vom Saal et al. 1997) were independently replicated and extended by Gupta (2000), which led to an editorial identifying "initial results confirmed" (Sheehan 2000).

Typically, within a laboratory, interesting findings are also followed by subsequent publications extending the prior findings; examples include the findings of BPA effects on β cells in the mouse pancreas (Alonso-Magdalena et al. 2005, 2006, 2008) and the effects of estrogenic chemicals and drugs on the developing mouse prostate that followed earlier findings (described above) from this same group (Timms et al. 2005; Richter et al. 2007b). In particular, independent replication by competent, respected scientists is the main criterion of acceptance of the findings as having been demonstrated to be reliable and having been validated by virtue of coming to the same conclusion using a variety of sophisticated techniques in multiple publications.

An important criticism of the approach taken by the U.S. FDA in its assessment of the now approximately 1,000 articles on BPA is that it appears to have made no attempt to connect the dots between replicated studies; instead, the U.S. FDA appears to have

assessed each study without regard to whether it had been confirmed by other studies.

Thus, collectively, many phases used to verify the reliability and validity of NIH-funded published research have been completely ignored by the U.S. FDA, whereas industry-funded GLP research is rarely, if ever, subject to these central requirements and yet is accepted by regulatory agencies as reliable and valid.

The U.S. FDA's misguided gold standard. In this light, the U.S. FDA's reliance upon GLP as the gold standard is scientifically misguided. Furthermore, U.S. FDA administrators are ignoring published critiques of the GLP studies it considers reliable and valid, such as the study by Tyl et al. (2002) and two coordinated studies conducted at the same time by Ashby et al. (1999) and Cagen et al. (1999). Each was an industry-funded study conducted using GLP. Each was harshly criticized in peer-reviewed publications by academic scientists and government panels [Center for the Evaluation of Risks to Human Reproduction (CERHR) 2007; NTP 2001; vom Saal and Hughes 2005; vom Saal and Welshons 2006]. Yet, the U.S. FDA and EFSA panels still assert that these studies represent the gold standard in toxicologic research.

Specifically, the studies of Cagen et al. (1999) and Ashby et al. (1999) were recently rejected by the NTP CERHR panel on BPA as unusable for consideration in its evaluation of the health hazards posed by BPA (CERHR 2007). Both the Ashby et al. (1999) and Cagen et al. (1999) studies reported finding no effect of their positive control [the estrogenic drug diethylstilbestrol (DES)] on any outcome, although these failures were not acknowledged by the authors in either article. In experimental science, the failure of a positive control to show an effect indicates the experiment failed, which is the conclusion reached by the CERHR panel (CERHR 2007).

The Tyl et al. 2002 study, which the U.S. FDA still accepts as a major study for determination of the safety of BPA (U.S. FDA 2008a, 2008b), was criticized by an NTP panel that met in 2000 to examine the low-dose issue (NTP 2001), as well as in subsequent publications (vom Saal and Hughes 2005; vom Saal and Welshons 2006), for using an insensitive rat (the CD-SD rat) that requires extremely high doses (≥ 50 $\mu\text{g}/\text{kg}/\text{day}$) of the potent estrogenic drug ethinylestradiol to show effects such as those examined in the study by Tyl et al. (2002). This dose of ethinylestradiol is > 100 times higher than the approximately 0.3 $\mu\text{g}/\text{kg}/\text{day}$ used by women in oral contraceptives. The fact that Tyl et al. (2002) adhered to GLP did not protect them from using insensitive animals. This led the NTP (2001) to state:

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.

Thus, when reviewed by other scientists, three prior major GLP studies of BPA have been found to be so flawed as to be useless for guiding regulatory agencies in decision making. A new GLP study has now been published by Tyl et al. (2008a). Close examination of this study also reveals fatal flaws which render it useless for regulatory purposes, even though it conforms to GLP.

Examples of Flaws Ignored by the U.S. FDA and EFSA in a Recent GLP Study of BPA

In summary, the flaws in Tyl et al. (2008a) are as follows:

- The high dose required for the positive control (estradiol) to cause an effect means the system used by Tyl et al. (2008a), at least in her laboratory, is relatively insensitive to exogenous estrogens and thus inappropriate for studying low-dose effects of estrogenic compounds such as BPA. The lack of response to low doses of estradiol or BPA in the Tyl laboratory is puzzling, in that the strain of mice used in these experiments (the CD-1 mouse) has been reported in > 20 other peer-reviewed publications to show adverse effects in response to very low doses of BPA (vom Saal 2008), as well as many other studies showing low-dose effects in response to the natural hormone estradiol, the estrogenic drugs ethinylestradiol and DES, and to other estrogenic chemicals.
- Tyl et al. (2008a) used insensitive, out-of-date protocols and assays that are incapable of finding many of the adverse effects reported by more sophisticated studies conducted by independent NIH-funded scientists as well as scientists funded by government agencies in other countries.
- In the specific case of testing for changes in prostate weight, Tyl et al. (2008a) reported an abnormally high prostate weight for control animals that exceeds by $> 70\%$ the prostate weights reported by other studies for animals of the same strain and similar age (e.g., Gupta 2000; Ruhlen et al. 2008). This suggests that the dissection procedures for the prostate in the Tyl laboratory included other nonprostatic tissues in the weight measurements, rendering them unusable for studying weight changes in the prostate in response to BPA or estradiol; neither chemical showed any effect on the selected end points, which directly contradicts other findings concerning opposite effects of low and high doses of estrogen on the prostate (Putz et al. 2001; Timms et al. 2005; vom Saal et al. 1997).

Aberrant insensitivity of CD-1 mouse to estrogens. Tyl et al. (2008a) used estradiol as a positive control. It was fed to female mice before and during pregnancy and lactation at 80–220 µg/kg/day; after weaning, estradiol was fed to offspring at doses of 80–100 µg/kg/day. Estradiol was used as a positive control because BPA is a man-made endocrine-disrupting estrogenic chemical.

Many published findings reporting effects of very low doses of positive control estrogens and BPA in CD-1 mice demonstrate that the CD-1 mouse was somehow rendered insensitive in the test system used by Tyl et al. (2008a). The fact that a dose of 100–200 µg/kg/day estradiol was necessary to show an effect of the positive control predicts that Tyl et al. (2008a) should not detect effects of BPA < 10–100 mg/kg/day, far above the low-dose range relevant to human exposures that was supposedly of interest.

For nuclear estrogen receptor-mediated effects via regulation of gene activity (nuclear estrogen receptors are transcription factors whose activity is regulated by binding to estrogen), prior studies have typically shown a 1,000-fold lower activity for BPA relative to estradiol or potent estrogenic drugs, including DES and ethinylestradiol. For example, Richter et al. (2007b) reported an increase in androgen receptor gene activity to estradiol at 1 pM (0.28 pg/mL) in fetal CD-1 mouse prostatic mesenchyme cells in primary culture, and the same response was found for BPA at 1,000 pM (228 pg/mL); the *in vitro* response to estradiol was predicted by the response of the prostate to increasing free serum estradiol from 0.2 to 0.3 pg/mL in male mouse fetuses via estradiol administration to the mother (vom Saal et al. 1997). Other research showed that a significant effect on development of the male reproductive system in CF-1 mice occurred at a maternal dose of 0.002 µg/kg/day ethinylestradiol (Thayer et al. 2001), similar to effects observed with 2–20 µg/kg/day BPA (vom Saal et al. 1998). The research of Honma et al. (2002) showed accelerated puberty in CD-1 (ICR) mice at a DES dose of 0.02 µg/kg/day (the positive control), and the same response to BPA occurred at 20 µg/kg/day, again revealing a 1,000-fold difference between the positive control estrogen and BPA.

There are many other examples of findings where a higher dose of BPA was required to cause the same effect as the positive control estrogen (estradiol, ethinylestradiol, or DES) in studies where the effects were mediated by the classical nuclear estrogen receptors, in contrast to the more recently discovered rapid signaling estrogen response system where BPA and these positive control estrogens have equal potency, as described above. In summary, CD-1 mice have been used by a large number of academic and government investigators and have been

reported in peer-reviewed publications to be sensitive to positive control estrogens within the range of human sensitivity based on *in vivo* and *in vitro* studies via the classical estrogen receptor α -mediated response mechanism. The CD-1 mouse is the animal model that has been used by the U.S. National Institute of Environmental Health Sciences (NIEHS) for decades, because it is considered the best animal model for predicting the effects of developmental exposure to estrogen in humans (Newbold 1995; Newbold et al. 2007).

The failure of traditional toxicologic studies conducted by Tyl et al. (2008a, 2008b) to detect the wide range of adverse effects of even relatively high doses of BPA or of low doses of estradiol that have been reported in numerous studies by academic and government scientists provides evidence that the GLP protocols established long ago by regulatory agencies to determine the toxicity of chemicals are inappropriate for detecting the endocrine-disrupting activities of chemicals such as BPA. Indeed, this was the premise of the congressional mandate in the Food Quality Protection Act (1996) for the U.S. EPA to establish a new set of assays for endocrine-disrupting chemicals, although this process has been systematically delayed and is > 8 years behind the congressionally mandated date of 2000 to have these new assays validated.

Citing Tyl et al. (2008a), the EFSA report on BPA (EFSA 2006) stated that “the positive control substance, 17 β -estradiol, resulted in reproductive and developmental toxicity.” This report failed to acknowledge that only a very high dose of the positive control was sufficient to elicit effects and that this meant that the experiments conducted in the Tyl laboratory were for some reason very insensitive to any estrogen and thus inappropriate for use in a study to examine low-dose estrogenic effects of BPA.

Based on the preliminary report released by the U.S. FDA regarding BPA (U.S. FDA 2008a), it appears that the U.S. FDA has followed the lead of the EFSA in its lack of understanding of the importance of the dose of the positive control estrogen required to cause adverse effects. The consequence is that the U.S. FDA has relied primarily on the study of Tyl et al. (2008a, 2008b), with the result that the U.S. FDA has assured Americans that BPA is safe at current human exposure levels.

Several factors might account for the insensitivity of the CD-1 mouse in the Tyl et al. studies (2008a, 2008b) conducted at Research Triangle Institute (RTI), a testing facility that conducted these (as well as previous) studies funded by the American Chemistry Council. One possibility is that the diet used in these studies may have interfered with the results. The feed used by Tyl et al. (2008a) in this experiment (Purina 5002) has been shown by

others to interfere with responses to exogenous estrogenic chemicals, blocking adverse effects documented on other diets. For example, a number of years ago, Thigpen et al. (2003) at the NIEHS recommended against the use of Purina 5002 in studies of endocrine-disrupting chemicals. Tyl et al. (2008a) measured some specific phytoestrogens in Purina 5002 feed by chemical analysis; however, in a report on NIH-sponsored meetings on this subject, Heindel and vom Saal (2008) pointed out that this is an insufficient control for total dietary estrogenic contaminants that can disrupt studies involving the effects of estrogenic chemicals.

A second possibility is that there are strain differences in sensitivity developed in the CD-1 mouse sold by the various Charles River Laboratories located in different regions. We consider this unlikely, because most laboratories regularly replace their CD-1 mouse breeder stock from Charles River Laboratories, and practices there make it unlikely that the sensitivity of this outbred stock to estrogens has changed dramatically over a very short period of time. Also, because RTI, where the Tyl studies were conducted, is very near the laboratories of the NIEHS, it is likely that the CD-1 mice used by these two programs were purchased from the same breeding facility.

Use of insensitive, out-of-date protocols and assays. Another serious concern about the two recent studies by Tyl et al. (2008a, 2008b) is the experimental approach used, thus raising questions about the validity of the studies. The study design used by Tyl et al. (2008a, 2008b) has been superseded by advances in both experimental design and analytical tools developed by NIH-funded scientists (and their counterparts in Europe and Asia) since the mid-1990s. The methods used by Tyl et al., primarily wet weight changes of tissues, gross histologic changes, and developmental landmarks such as vaginal opening, were established procedures by the 1950s. Thus, a major limitation of the Tyl studies is the failure to measure more meaningful and sensitive end points in order to detect the effects of low-dose BPA exposure, which are often not macroscopic in nature. Indeed, in 2001, the director of the reproductive division of the National Health and Environmental Effects Research Laboratory at the U.S. EPA stated that the inconclusive results concerning effects of BPA on reproductive toxicology can only be solved by understanding the mechanisms (Triendl 2001). With current GLP standards it is not possible to study mechanisms because they still rely on out-of-date assays.

As one example of a comparison between the approach by Tyl et al. (2008a) and independent government-funded academic scientists, extensive research has been conducted by Soto et al. (2008) and by other independent academic and government scientists

describing effects of exposure of female mice and rats to very low doses of BPA during perinatal development on the mammary glands (Jenkins et al. 2009). Although Tyl et al. (2008a) reported no low-dose effects of BPA on the mammary glands using conventional histologic analysis, there have been consistent findings of adverse effects of low doses of BPA from studies that used more sophisticated and sensitive analysis of whole mounted mammary glands to facilitate detection of microscopic lesions, coupled with immunostaining for regulatory proteins as well as techniques for determination of aberrant gene expression associated with progression to cancer. These peer-reviewed studies have reported detecting changes during embryonic development of mammary glands as well as abnormalities detected during adolescence through adulthood that are indicative of mammary gland cancer as well as other developmental abnormalities (Colerangle and Roy 1997; Durando et al. 2007; Jenkins et al. 2009; LaPensee et al. 2008; Markey et al. 2001, 2005; Moral et al. 2008; Munoz-de-Toro et al. 2005; Murray et al. 2007; Nikaido et al. 2004; Vandenberg et al. 2006, 2007b; Wadia et al. 2007).

Similar to the findings for the mammary gland, Ogura et al. (2007) reported that if tissues were analyzed by conventional histologic methods (staining with hematoxylin and eosin), prenatal exposure to low doses of BPA or DES showed no effects on prostate development, whereas if the sections were analyzed using antibodies that identified basal cells and basal cell squamous metaplasia, then significant effects were revealed. Squamous metaplasia of basal cells indicates abnormal proliferation and function of the prostate stem cell population that is thought to transform into neoplastic cells; Ho et al. (2006) reported that neonatal exposure to very low doses of BPA caused 100% of male rats to develop high-grade prostatic intraepithelial neoplastic lesions later in life. All of these studies were rejected by the U.S. FDA as not adequate for making regulatory decisions about the safety of BPA. Instead, the U.S. FDA relied upon Tyl et al. (2008a), even though the study used techniques that Ogura et al. (2007) showed lacked the sensitivity of 21st century experimental approaches.

Although findings regarding changes in brain structure, brain chemistry, and behavior represent the largest portion of the literature on low-dose BPA, Tyl et al. (2008a) did not examine any neurobehavioral end points. The NTP (2008) and the NIEHS conference consensus reports (vom Saal et al. 2007) both indicated concern about neurobehavioral effects of low doses of BPA. Thus, the absence of studies that included neurobehavioral end points is a glaring omission of Tyl et al. (2008a, 2008b).

Flawed prostate dissection. Data presented by Tyl et al. (2008a) raise questions about the adequacy of techniques used in their BPA studies. Specifically, Tyl et al. (2008a) reported that the prostate in 3.5-month-old control male CD-1 mice weighed > 70 mg [see Table 3 in Tyl et al. (2008a) for data on F₁ retained males]. This average control weight contrasts sharply with those reported from other laboratories. Specifically, the weight of the prostate in 2- to 3-month-old CD-1 mice using the dissection technique based on both Rublen et al. (2008) and Gupta (2000) and at the NIEHS (Newbold RR, personal communication) is about 40 mg. Several studies have reported that prenatal exposure to very low doses of BPA and positive control estrogens increased prostate size, prostatic androgen receptors, and prostate androgen receptor gene activity (Gupta 2000; Richter et al. 2007b; Thayer et al. 2001; Timms et al. 2005; vom Saal et al. 1997), but the enlarged prostate of experimental animals exposed to BPA in these laboratories weighed less than the prostates in the control animals of Tyl et al. (2008a). This raises serious questions about the procedures and/or animals used by Tyl et al. The weight of prostate reported by Tyl et al. (2008a) suggests that the technique used for dissecting the prostate resulted in non-prostatic tissue being weighed along with prostate. The seminal vesicle, coagulating gland, and dorsolateral prostate all merge together where the ejaculatory ducts enter the urethra, and there are also fat deposits on the prostate. This poses a challenge for those without proper training in distinguishing these different tissues during dissection in mice.

Alternatively, as male rodents age, they are prone to develop prostatitis. Although this inflammatory disease leads to an increase in prostate size and could thus account for the very large prostate weights reported by Tyl et al. (2008a), anyone familiar with the appearance of prostatitis would detect this abnormality upon histologic examination, which Tyl et al. (2008a) supposedly conducted. Also, prostatitis is rare in young-adult mice or rats (Cowin et al. 2008), and the size of the prostates in the Tyl et al. (2008a) study were similar to those for middle-aged and old male mice.

The findings regarding effects of BPA on the prostate presented by Tyl et al. (2008a) are thus suspect and cannot be used as evidence that other earlier studies (Gupta 2000; Timms et al. 2005; vom Saal et al. 1997) are not replicable. Given these problems in prostate weight measurements, it is not surprising that even very high doses of BPA or estradiol reported by Tyl et al. (2008a) had no effect on the prostate, in sharp contrast to other studies that showed stimulation of the prostate at low doses of estrogen and inhibition at high doses (Putz et al. 2001; Timms et al. 2005).

In addition to the problem associated with the high prostate weight reported by Tyl et al. (2008a), in a separate measurement the authors combined the anterior prostate (coagulating gland) and seminal vesicle, presenting these two organs as one combined outcome measure. This is wrong and misleading. The coagulating glands emerge as the anterior ducts of the prostate from the dorsocranial region of the urogenital sinus, whereas the seminal vesicles bud from the proximal region of the Wolffian ducts. Elevated estrogen is associated with an increase in prostate size associated with an increase in prostate androgen receptors, whereas a decrease in seminal vesicle size is associated with a reduction in 5 α -reductase, an enzyme that converts testosterone to the more potent androgen 5 α -dihydrotestosterone (Nonneman et al. 1992). Low doses of BPA have been shown to decrease the size of organs that differentiate from the embryonic Wolffian ducts (epididymides and seminal vesicles) while increasing the size of regions of the prostate that develop from the urogenital sinus (vom Saal et al. 1998). Combining these different organs (it is technically not difficult to separate them) was thus inappropriate because they develop from different embryonic tissues that show markedly different responses to estrogenic chemicals during development. In fact, Ogura et al. (2007) reported that the anterior prostate (coagulating glands) showed the greatest expression of ER- α , and also showed the most pronounced indication of basal cell squamous metaplasia in response to developmental exposure to low doses of DES and BPA relative to other regions of the prostate.

Conclusions

Because the control data of Tyl et al. (2008a) were not consistent with the prior published literature for prostate weight of young-adult CD-1 male mice and because their methods were inappropriate for revealing an extensive body of adverse effects detected using more sophisticated approaches, we deem the findings by Tyl et al. to be invalid. Hundreds of studies show adverse effects of BPA in animals, with many conducted at concentrations equivalent to current human levels of BPA exposure; thus, it is unlikely that academic scientists would bother to replicate the outdated approaches used by Tyl et al. (2008a, 2008b). This lack of replication is typical of GLP studies, which tend to involve unnecessarily large numbers of animals [Tyl et al. (2002) used > 8,000 rats], and reliability appears to be accepted because of the numbers of animals that were used. Although using excessive numbers of animals is accepted as good science by the U.S. FDA, the use of arbitrarily large numbers of animals per group (> 20 animals per treatment group is common) actually violates guidelines in the NIH *Guide for the*

Care and Use of Laboratory Animals (Institute of Laboratory Animal Research 1996) that govern research conducted by academic and government scientists. For research with animals to be approved by any university animal care and use committee, group sizes must be based on power analysis conducted using historic data. Based on this criterion in the NIH Guide, all of the studies by Tyl et al. were significantly over powered and thus in direct violation of federal guidelines for conducting animal research, a fact about which U.S. FDA regulators seem unaware.

Each of the four main industry-funded GLP studies of BPA (Ashby et al. 1999; Cagen et al. 1999; Tyl et al. 2008a, 2008b) is flawed and not appropriate for use in setting health standards. Clearly, meeting GLP standards is not a guarantee of reliable or valid science. It is of great concern that the U.S. and EU regulatory communities are willing to accept these industry-funded, antiquated, and flawed studies as proof of the safety of BPA while rejecting as invalid for regulatory purposes the findings from a very large number of academic and government investigators using 21st-century scientific approaches. The basis for these decisions by U.S. and EU regulatory agencies should be thoroughly investigated, particularly since the NTP (2008) concluded that BPA exposure to human infants was in the range shown to cause harm in experimental animals and since both the Canadian Ministry of Health and the Ministry of the Environment recently concluded that BPA was a toxic chemical (Environment Canada 2008).

Problems inherent with reliance on GLP as the standard for choosing data are compounded by the process used by federal agencies to determine membership on science advisory panels. Leading experts qualified by specific experience on the chemical or end points under consideration are often specifically excluded from membership. For example, the U.S. FDA's BPA review panel was identified as an expert panel, when in fact the panel was composed largely of scientists lacking any experience in research with BPA. This process, which appears to consider almost any scientist knowledgeable about a chemical to create bias, makes it vastly more difficult for the panel to integrate scientific data from the relevant literature, especially since, as with BPA, there are almost 1,000 relevant studies and the review panel is provided with very little time to become knowledgeable about the details. It means that the depth of knowledge present on this and similarly constituted government regulatory agency panels is unlikely to be sufficient to subject draft assessments to the scrutiny that peer review by experts normally entails. Combined with reliance on GLP data, this process has a high potential to yield flawed assessments that jeopardize public health.

We are not suggesting that GLP should be abandoned as a requirement for industry-funded studies. We object, however, to regulatory agencies implying that GLP indicates that industry-funded GLP research is somehow superior to NIH-funded studies that are not conducted using GLP. This argument demonstrates a lack of understanding of the profound difference between the use of replication as a mechanism to assess reliability and the methods used to assess validity for peer-reviewed published academic studies, whereas GLP was instituted with the expectation that this type of verification would not occur.

Public health decisions should be based on studies using appropriate protocols and the most sensitive assays. They should not be based on criteria that include or exclude data depending on whether or not the studies use GLP. Simply meeting GLP requirements is insufficient to guarantee scientific reliability and validity.

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Polycarbonate Bottle Use and Urinary Bisphenol A Concentrations

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BACKGROUND: Bisphenol A (BPA) is a high-production-volume chemical commonly used in the manufacture of polycarbonate plastic. Low-level concentrations of BPA in animals and possibly in humans may cause endocrine disruption. Whether ingestion of food or beverages from polycarbonate containers increases BPA concentrations in humans has not been studied.

OBJECTIVES: We examined the association between use of polycarbonate beverage containers and urinary BPA concentrations in humans.

METHODS: We conducted a nonrandomized intervention of 77 Harvard College students to compare urinary BPA concentrations collected after a washout phase of 1 week to those taken after an intervention week during which most cold beverages were consumed from polycarbonate drinking bottles. Paired *t*-tests were used to assess the difference in urinary BPA concentrations before and after polycarbonate bottle use.

RESULTS: The geometric mean urinary BPA concentration at the end of the washout phase was 1.2 µg/g creatinine, increasing to 2.0 µg/g creatinine after 1 week of polycarbonate bottle use. Urinary BPA concentrations increased by 69% after use of polycarbonate bottles ($p < 0.0001$). The association was stronger among participants who reported $\geq 90\%$ compliance (77% increase; $p < 0.0001$) than among those reporting $< 90\%$ compliance (55% increase; $p = 0.03$), but this difference was not statistically significant ($p = 0.54$).

CONCLUSIONS: One week of polycarbonate bottle use increased urinary BPA concentrations by two-thirds. Regular consumption of cold beverages from polycarbonate bottles is associated with a substantial increase in urinary BPA concentrations irrespective of exposure to BPA from other sources.

KEY WORDS: biomarkers, bisphenol A, endocrine disruptors, human, polycarbonate plastic. *Environ Health Perspect* 117:1368–1372 (2009). doi:10.1289/ehp.0900604 available via <http://dx.doi.org/> [Online 12 May 2009]

The endocrine-disrupting chemical bisphenol A (BPA) has recently garnered heightened attention because of widespread human exposure and disruption of normal reproductive development in laboratory animals [Center for the Evaluation of Risks to Human Reproduction (CERHR) 2008; Chapin et al. 2008; Goodman et al. 2006; European Union 2003; vom Saal and Hughes 2005]. BPA is thought to disrupt normal cell function by acting as an estrogen agonist (Wozniak et al. 2005) as well as an androgen antagonist (Lee et al. 2003). In animal studies, prenatal and neonatal exposure to BPA has been linked to early onset of sexual maturation (Howdeshell et al. 1999), altered development and tissue organization of the mammary gland (Markey et al. 2001), induction of preneoplastic mammary gland (Durando et al. 2007) and reproductive tract lesions (Newbold et al. 2007), increased prostate size (Timms et al. 2005), and decreased sperm production (vom Saal et al. 1998) in offspring. Most recently, exposure to BPA has also been associated with chronic disease in humans, including cardiovascular disease, diabetes, and serum markers of liver disease (Lang et al. 2008).

Orally administered BPA is rapidly metabolized by glucuronidation during first-pass metabolism, with a biological half-life

of approximately 6 hr and nearly complete elimination within 24 hr (Volkel et al. 2002). However, because of continuous and widespread exposure, $> 92\%$ of the 2,517 participants ≥ 6 years of age in the U.S. 2003–2004 National Health and Nutrition Examination Survey (NHANES) had detectable concentrations of BPA in their urine (Calafat et al. 2008). The geometric mean (GM) urinary BPA concentration in that study was 2.6 µg/L (2.6 µg/g creatinine), and the 95th percentile was 15.9 µg/L (11.2 µg/g creatinine).

An important source of human exposure is thought to be the ingestion of food and drink that has been in contact with epoxy resins or polycarbonate plastics (Kang et al. 2006). Polycarbonate is a durable, lightweight, and heat-resistant plastic, making it popular for use in plastic food and beverage containers. Indeed, nearly three-fourths of the 1.9 billion pounds of BPA used in the United States in 2003 was used for the manufacture of polycarbonate resin (CERHR 2008). Other common uses of BPA include the manufacture of epoxy resins used as composites and sealants in dentistry and in the lacquer lining of aluminum food and beverage cans (CERHR 2008; European Union 2003).

Laboratory studies have demonstrated that biologically active BPA is released from

polycarbonate bottles after simulated normal use (Brede et al. 2003; Le et al. 2008). High temperatures as well as acidic and alkali solutions cause polymer degradation via hydrolysis, resulting in increased BPA migration. After incubation for 8, 72, and 240 hr in food-simulating solvents (10% ethanol at 70°C and corn oil at 100°C), mean BPA migration increased with incubation time (Onn Wong et al. 2005). After a sequence of washing and rinsing, Le et al. (2008) found that new polycarbonate bottles leached 1.0 ± 0.3 µg/mL BPA (mean \pm SD) into the bottle content after incubation at room temperature for 7 days. Although exposure to boiling water increased the rate of BPA migration up to 55-fold, used bottles did not leach significantly more BPA than new ones. However, other studies have found that higher concentrations of BPA leach from used polycarbonate plastic than from new. BPA has been observed to leach from polycarbonate animal cages after 1 week of incubation at room temperature, with higher levels of migration from used versus new cages (Howdeshell et al. 2003). Similarly, after incubation in 100°C water for 1 hr, the amount of BPA leached from baby bottles subjected to simulated use (including dishwashing, boiling, and brushing into the bottle) exceeded the amount that leached from new baby bottles (Brede et al. 2003).

Recently, some polycarbonate bottle manufacturers voluntarily eliminated BPA from their products (Nalgene Outdoor 2008), and several retailers withdrew polycarbonate bottles from

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their stores altogether (Mui 2008). Canada has imposed a ban on the use of BPA in polycarbonate baby bottles in order to reduce exposure of infants to BPA (Health Canada 2008), and similar legislation is being considered by several U.S. states (Austen 2008). However, such actions have been largely preemptive, as no epidemiologic study has evaluated the physiologic consequences of polycarbonate bottle use. Therefore, we studied the impact of cold beverage consumption from polycarbonate bottles on measurable urinary BPA concentrations among a Harvard College population. We also measured exposure to the phenols triclosan (TCS), methyl paraben (MePB), propyl paraben (PrPB), and benzophenone-3 (BP-3), which occurs mainly through the use of personal care products. Therefore, because exposure of these chemicals is considered unrelated to polycarbonate bottle use, we assessed their association with polycarbonate bottle use as a negative control.

Materials and Methods

Study population. We recruited Harvard College students in April 2008 via e-mails to freshman dormitory, upperclass house, and student organization mailing lists. Students were directed to a survey website, where they provided contact information and indicated their availability for the study dates. Participant instructions and informed consent forms were also made available. Students at least 18 years of age who were available for the entire study period were considered eligible and were invited to an introductory meeting. The 89 students who attended the meeting returned their signed informed consent forms, provided demographic information (age, sex, ethnicity), and received two stainless steel bottles. Seven participants withdrew from the study before completing the washout phase, and five participants withdrew after completing the washout phase but before completion of the intervention phase. Participants who withdrew were similar to those who completed the study in terms of age (median, 19 years; range, 18–22 years) but were slightly more likely to be female (66.7%) than students who completed the entire study. A total of 77 participants completed the study. A \$25 compensation was provided only upon completion of the study. The study was approved by the Human Studies Institutional Review Board of Harvard University. The involvement of the Centers for Disease Control and Prevention (CDC) laboratory was limited and was determined not to constitute engagement in human subjects research.

Study design. The study began with a 7-day washout phase designed to minimize exposure to BPA by limiting the consumption of cold beverages to those contained in stainless steel bottles. Because orally administered BPA is

rapidly excreted (Volkel et al. 2002), we considered a 1-week washout period sufficient. We provided participants with two stainless steel bottles and advised them to drink all cold beverages from the stainless steel bottles and avoid drinking water from #7 polycarbonate plastic cold water dispensers available in college dining halls. Participants donated urine on their choice of 2 of 3 final days of the washout phase. Urine donation took place between 1700 and 2000 hours on two of the days, and between 1600 and 1900 hours on the third day. Two polycarbonate bottles were distributed to each participant on the second day of urine donation during the washout phase. Participants were advised to begin drinking all cold beverages from the polycarbonate bottles (intervention week) immediately. Urine was donated again on the participant's choice of 2 of 3 final days of the week of polycarbonate bottle use between 1700 and 2000 hours. On the final day of urine donation, participants completed a brief questionnaire in which they estimated their percentage compliance during the week in which they were asked to drink cold beverages from the polycarbonate bottles.

Stainless steel bottles (27 fluid ounces, with #5 polypropylene loop cap) were obtained from Kleen Kanteen (763332017107; Chico, CA). Polycarbonate bottles [Nalgene 32 fluid ounce, Lexan narrow mouth (#53175), and Lexan wide mouth (#53107)] were obtained from Karst Sports (Shinnston, WV). All participants were permitted to keep the bottles used in the study.

Urine sample collection. Urine was collected in a polypropylene container, aliquoted, and frozen at -20°C . After study completion, samples were defrosted at 4°C overnight and vortexed; equal volumes of the two samples from each phase of the study were then combined and aliquoted. We shipped aliquots of samples (blinded to those performing laboratory analyses) on dry ice overnight to the CDC for measuring BPA and other urinary phenol concentrations; samples were also taken to N. Rifai (Children's Hospital, Boston, MA) for analysis of urinary creatinine.

Urinary phenol concentrations. Total urinary concentrations (free plus conjugated species) of BPA and the other four phenols were determined using online solid-phase extraction coupled to isotope dilution high-performance liquid chromatography (HPLC)-tandem mass spectrometry (MS/MS) on a system constructed from several HPLC Agilent 1100 modules (Agilent Technologies, Wilmington, DE) coupled to a triple quadrupole API 4000 mass spectrometer (Applied Biosystems, Foster City, CA) (Ye et al. 2005). First, 100 μL urine was treated with β -glucuronidase/sulfatase (*Helix pomatia*; Sigma Chemical Co., St. Louis, MO) to hydrolyze conjugated species of the phenols. The phenols were then

retained and concentrated on a C18 reversed-phase size-exclusion solid-phase extraction column (Merck KGaA, Darmstadt, Germany), separated from other urine matrix components using a pair of monolithic HPLC columns (Merck KGaA), and detected by negative ion-atmospheric pressure chemical ionization-MS/MS. The limits of detection (LODs) in a 0.1-mL urine sample were 0.4 $\mu\text{g/L}$ (BPA and BP-3), 0.2 $\mu\text{g/L}$ (PrPB), 1.0 $\mu\text{g/L}$ (MePB), and 2.3 $\mu\text{g/L}$ (TCS). Low-concentration (~ 4 to ~ 25 $\mu\text{g/L}$) and high-concentration (~ 10 to ~ 65 $\mu\text{g/L}$) quality-control materials, prepared with pooled human urine, were analyzed with standard, reagent blank, and unknown samples (Ye et al. 2005). Creatinine was measured by a rate-blanked method using the Hitachi 917 analyzer and Roche Diagnostics reagents (both from Roche Diagnostics, Indianapolis, IN).

Statistical analysis. Urinary phenol concentrations were normalized for dilution using the formula $100 \times$ urinary phenol concentration (micrograms per liter) \div creatinine concentration (milligrams per deciliter). Creatinine-adjusted phenol concentrations (micrograms per gram creatinine) were not normally distributed and were therefore log-transformed. Phenol concentrations $<$ LOD were assigned a value equal to one-half the LOD (Hornung 1990) prior to creatinine adjustment.

We calculated GMs for creatinine-corrected concentrations. We used paired *t*-tests to examine the association between log-transformed urinary creatinine-adjusted phenol concentrations and drinking-container assignment overall and within subsets defined by percent compliance during the intervention phase (\geq median and $<$ the median). When the participant reported compliance as a range, we used the mean. Two sample *t*-tests were used to make comparisons between the strata defined by percent compliance.

Results

The study population included 77 subjects who ranged in age from 18 to 23 years, with a median of 19 years (Table 1). On the basis

Table 1. Characteristics of 77 Harvard College students enrolled in a nonrandomized intervention study assessing changes in urinary phenol concentrations associated with use of polycarbonate drinking containers.

Characteristic	No. (%)
Sex	
Male	41 (53.2)
Female	36 (46.8)
Ethnicity	
Caucasian	30 (39.0)
Asian	38 (49.3)
African American	5 (6.5)
Hispanic	4 (5.2)
Percent compliance [median of proportion (range)]	90 (50–100)
Age, years [median (range)]	19 (18–23)

of self-reported data, we categorized race/ethnicity into four groups: Caucasian, Asian, African American, and Hispanic. Thirty participants (39.0%) were Caucasian, 38 were of Asian descent (49.4%), 5 were African American (6.5%), and 4 were Hispanic (5.2%). Forty-one subjects were male (53.3%). Protocol compliance for the week in which participants drank from polycarbonate bottles ranged from 50% to 100% but was generally high, with a median of 90%.

Nine samples (11.7%) from the washout week and three samples (3.9%) from the intervention week (period in which participants drank from polycarbonate bottles) had BPA concentrations < LOD. BP-3 and MePB were detected in all participants, and PrPB was detected in all but one participant each week. TCS was detected in 75.3% of the samples taken at the end of the washout week and in 74.0% of the samples collected after the intervention week. The GM concentration of BPA was 1.3 µg/L (1.2 µg/g creatinine) during the washout period and 2.1 µg/L (2.0 µg/g creatinine) during the intervention week (Table 2). GM concentrations for the washout phase and intervention week were 46.1 and 66.8 µg/g creatinine for BP-3; 51.3 and 48.4 µg/g creatinine for MePB; 8.4 and 8.8 µg/g creatinine for PrPB; and 15.5 and 17.3 µg/g creatinine for TCS, respectively.

Table 3 presents results from paired *t*-tests comparing urinary BPA concentrations in weeks 1 and 2. Urinary BPA concentrations increased by 69% after polycarbonate bottle use. We observed a larger difference between the intervention and washout weeks in the stratum with intervention compliance ≥ 90% (77% increase; *p* < 0.0001) relative to the stratum with compliance < 90% (55%; *p* = 0.03); however, the strata were not significantly different from each other (*p* = 0.54). Of the other phenols, only urinary BP-3 concentration was associated with polycarbonate bottle use, with relatively higher concentrations observed after use of polycarbonate bottles (45% increase; *p* = 0.001). A slightly larger change in BP-3 concentration was observed in the less

Table 2. GM concentrations of phenols (µg/creatinine) after washout and intervention.

Phenol	Week of study	GM (95% CI)
BPA	Washout	1.2 (1.0–1.4)
	Intervention	2.0 (1.7–2.4)
BP-3	Washout	46.1 (30.6–69.5)
	Intervention	66.8 (42.3–105.5)
MePB	Washout	51.3 (37.3–70.7)
	Intervention	48.4 (36.2–64.8)
PrPB	Washout	8.4 (5.4–12.9)
	Intervention	8.8 (5.8–13.1)
TCS	Washout	15.5 (9.5–25.3)
	Intervention	17.3 (10.7–28.1)

Concentrations (µg/L) < LOD were recorded as 1/2 LOD, which is 0.2 for BPA and BP-3; 1.15 for TCS; 0.5 for MePB; and 0.1 for PrPB.

compliant stratum (64% increase; *p* = 0.01) relative to the more compliant stratum (36% increase; *p* = 0.04); however, this difference was not statistically significant (*p* = 0.42).

Discussion

Several previous studies have demonstrated that biologically active BPA is released from polycarbonate bottles into the bottle content after simulated normal use (Brede et al. 2003; Le et al. 2008). However, to our knowledge, the present study is the first to quantify the corresponding increase in urinary BPA concentrations after use of polycarbonate drinking bottles. Thus, this study suggests that BPA-containing drinking vessels release sufficient amounts of BPA into the bottle content to significantly raise the amount of BPA excreted in urine in humans who drink from these bottles. Specifically, in this study of 77 Harvard College students, urinary BPA concentrations were higher when participants consumed the majority of cold beverages from polycarbonate bottles compared with a washout phase in which polycarbonate bottles were avoided. This statistically significant increase was observed despite background BPA exposure from other sources, which was not assessed nor controlled in this study. This association persisted after stratification by self-reported compliance during the intervention week, with a nonsignificantly larger difference between intervention and washout phase urinary BPA concentrations among participants reporting higher percent compliance. Of interest, the urinary BPA concentrations reported for this group of students (both before and after the intervention) were similar to those reported for the U.S. general population (Calafat et al.

2008) and selected populations in Southeast Asia (Kim et al. 2003; Matsumoto et al. 2003; Ouchi and Watanabe 2002; Yang et al. 2003).

Because of BPA's short half-life and rapid elimination (Volkel et al. 2002), carryover of ingested BPA between the washout phase and intervention phase was considered unlikely. It is possible that certain subject characteristics may have varied between the 2 weeks, producing a period effect that was unaccounted for by our analyses. We considered this improbable because of the lack of variability in the routine of undergraduate students, who attended the same classes and ate in the same campus dining halls each week. Additionally, the similarity of observed urinary BPA concentrations to national levels suggests that subjects were exposed to typical amounts of BPA from other sources during both weeks. Moreover, fatigue and the participants' exposure to mass media concerning the leaching of BPA from polycarbonate bottles might have induced better compliance during the washout phase than the intervention phase, thus leading to an underestimate of the impact of polycarbonate bottle use on urinary BPA concentrations. It is also possible that participants may have modified their behavior during the week of polycarbonate bottle use to reduce BPA exposure from other sources. However, other sources of BPA exposure have not been well publicized, and any reduction in exposure to other sources of BPA during the intervention week would have reduced the observed effect estimate.

We used spot urine samples for convenience; however, disadvantages of this method include interperson variability in BPA concentration and variability in the volume of urine (Barr et al. 2005). Two equal-volume

Table 3. Percent change in urinary concentrations of phenols associated with 1-week use of polycarbonate drinking containers.

Phenol	Percent change (95% CI)	<i>p</i> -Value	<i>p</i> for heterogeneity
BPA			
Overall	69 (40 to 102)	< 0.0001	
≥ 90% compliance	77 (45 to 117)	< 0.0001	
< 90% compliance	55 (6 to 127)	0.03	0.54
BP-3			
Overall	45 (16 to 81)	0.001	
≥ 90% compliance	36 (2 to 80)	0.04	
< 90% compliance	64 (11 to 142)	0.01	0.42
MePB			
Overall	-6 (-25 to 18)	0.60	
≥ 90% compliance	17 (-10 to 51)	0.24	
< 90% compliance	-34 (-56 to 0)	0.05	0.01
PrPB			
Overall	5 (-24 to 44)	0.77	
≥ 90% compliance	15 (-23 to 70)	0.49	
< 90% compliance	-10 (-49 to 59)	0.70	0.46
TCS			
Overall	12 (-17 to 50)	0.46	
≥ 90% compliance	11 (-18 to 50)	0.50	
< 90% compliance	17 (-39 to 126)	0.62	0.88

Concentrations (µg/L) < LOD were recorded as 1/2 LOD, which is 0.2 for BPA and BP-3; 1.15 for TCS; 0.5 for MePB; and 0.1 for PrPB. Twenty-eight participants reported < 90% compliance over intervention week, 48 participants reported ≥ 90% compliance, and compliance was missing for one participant.

samples from each week were combined to minimize day-to-day variability. Additionally, we collected all urine samples in the evening, minimizing variability related to time of day (Mahalingaiah et al. 2008). Concern regarding interperson variability is also mitigated by recent findings that a single urinary BPA concentration was predictive of long-term exposure on a scale of weeks to months (Mahalingaiah et al. 2008). Urinary BPA concentrations were creatinine-adjusted to account for variability in urine dilution. Overall, the results obtained after the analysis with and without correction of the urinary dilution were fairly similar. However, failure to control for urinary creatinine concentrations resulted in a greater degree of within-person variation and, subsequently, decreased precision, as evidenced by wider 95% CIs. For this reason, we have presented only the creatinine-adjusted results.

To account for the possibility of a chance finding, we also compared the impact of polycarbonate bottle use on several phenols not thought to be associated with polycarbonate bottle use. As expected, we observed no difference for MePB, PrPB, or TCS, although urinary concentrations of BP-3 were higher after polycarbonate bottle use. However, after stratification by percent compliance during the intervention week, the association for BP-3 was stronger in the less compliant group. By contrast, the association between BPA and polycarbonate bottle use was stronger in the more compliant group, suggesting that BPA may leach from polycarbonate bottles. We found BPA and BP-3 to be strongly correlated: The Pearson correlation coefficients between BP-3 and BPA were 0.38 ($p = 0.0008$) and 0.43 ($p = 0.0001$) during the washout week and intervention week, respectively. Although this study was not designed to look at other sources of BPA, or any sources of the other phenols, we hypothesize that the strong correlation observed between BPA and BP-3 could be the result of a shared source or behavior. We are not aware of the presence of BP-3—a common sunscreen agent in personal care products—in polycarbonate plastic, although it can also be used as ultraviolet stabilizer in plastic surface coatings for food packaging to prevent polymer or food photodegradation (Suzuki et al. 2005). However, because sources and routes of exposure for many of these compounds are not yet known, it is possible that BPA and BP-3 are used in a common product that has not yet been identified. An alternative explanation is that students who participated in the most outdoor physical activity drank the most fluid from their bottles and also applied the most sunscreen, potentially increasing both BPA and BP-3 levels.

Our study population included a high proportion of Asian and Caucasian participants, and our participants were young. However,

there is no obvious reason why the results of our study should not apply to other ethnicities and age groups. Furthermore, the use of polycarbonate bottles is very popular among college students, making this an especially relevant population to study. Although we assessed the effect of the exclusive use of polycarbonate plastic bottles as beverage containers, a proportionate increase in urinary BPA would be expected among individuals who use polycarbonate plastic bottles in combination with other beverage containers. Children have been found to have higher urinary BPA concentrations than adolescents and adults (Calafat et al. 2008), consistent with animal evidence of reduced glucuronidation in fetuses and neonates (Matsumoto et al. 2002). Thus, because of their reduced ability to clear BPA, we predict that children would have higher urinary BPA concentrations due to use of polycarbonate plastic bottles relative to the study population.

The major strength of this study is the non-randomized intervention design. We compared urinary BPA concentrations within each participant, which precluded confounding by subject characteristics that remain constant over time. Although within-person confounding was possible, it is unlikely that unmeasured confounding could account for the large effect estimate we observed. The large increase in mean urinary BPA concentration after regular use of polycarbonate bottles suggests that the systematic BPA variation in the two study phases was by far greater than any random variation due to BPA ingestion from other sources.

Furthermore, we assessed the impact of polycarbonate bottle use in a normal use setting. The present study could be considered a conservative estimate of true use, as students did not have access to dishwashers and were instructed to use their containers for cold beverages only, whereas the storage of hot liquids is common, especially in outdoor recreation settings. Because heating is thought to increase the amount of BPA leached from the polycarbonate (Le et al. 2008), we would anticipate higher urinary BPA concentrations after ingestion of hot beverages stored in the same bottles.

Conclusions

To our knowledge, this is the first study to assess the impact of polycarbonate drinking bottle use on urinary BPA concentrations. Despite within-person variability resulting from other sources of BPA exposure, a measurable increase in urinary BPA resulted from only 1 week of exposure to beverages contained in polycarbonate bottles. Replication of this study in other populations may help to inform public health policy regarding the use of BPA in polycarbonate food and beverage containers.

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Does Rapid Metabolism Ensure Negligible Risk from Bisphenol A?

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BACKGROUND: Bisphenol A (BPA) risks are being evaluated by many regulatory bodies because exposure is widespread and the potential exists for toxicity at low doses.

OBJECTIVE: We evaluated evidence that BPA is cleared more rapidly in humans than in rats in relation to BPA risk assessment.

DISCUSSION: The European Food Safety Authority (EFSA) relied on pharmacokinetic evidence to conclude that rodent toxicity data are not directly relevant to human risk assessment. Further, the EFSA argues that rapid metabolism will result in negligible exposure during the perinatal period because of BPA glucuronidation in pregnant women or sulfation in newborns. These arguments fail to consider the deconjugation of BPA glucuronide *in utero* by β -glucuronidase, an enzyme that is present in high concentrations in placenta and various other tissues. Further, arylsulfatase C, which reactivates endogenous sulfated estrogens, develops early in life and so may deconjugate BPA sulfate in newborns. Biomonitoring studies and laboratory experiments document free BPA in rat and human maternal, placental, and fetal tissues, indicating that human BPA exposure is not negligible. The pattern of these detections is consistent with deconjugation in the placenta, resulting in fetal exposure. The tolerable daily intake set by the EFSA (0.05 mg/kg/day) is well above effect levels reported in some animal studies.

CONCLUSION: This potential risk should not be dismissed on the basis of an uncertain pharmacokinetic argument. Rather, risk assessors need to decipher the BPA dose response and apply it to humans with comprehensive pharmacokinetic models that account for metabolite deconjugation.

KEY WORDS: β -glucuronidase, bisphenol A, endocrine disruption, fetus, glucuronidation, metabolism, neonate. *Environ Health Perspect* 117:1639–1643 (2009). doi:10.1289/ehp.0901010 available via <http://dx.doi.org/> [Online 14 July 2009]

Few current or past risk assessment issues are as challenging as those raised by bisphenol A (BPA). There is widespread BPA exposure to the general public, including pregnant women and infants, and the chemical is in the class of environmental hormones for which risk assessment approaches are still developing. Given that BPA is one of a large number of estrogenic chemicals to which humans are frequently exposed, this chemical represents something of a test case. Increasing the stakes is evidence for low-dose effects within the range of human exposure for end points that have implications for reproductive health and cancer. However, this evidence is in dispute, with major scientific and regulatory bodies disagreeing over BPA's low-dose risks. Health Canada (2008) calls it a hazardous substance and has banned it from baby bottles. In contrast, the U.S. Food and Drug Administration and the European Food Safety Authority (EFSA) have found no immediate cause for concern and are not taking action to limit exposure.

Other recent reviews and commentaries have focused on the evidence of harm from low-dose exposure (Bucher 2009; Myers et al. 2009; Tyl 2008); we do not address those data here. Rather, we focus on an element that has not received critical attention: the claim raised by the EFSA that rapid metabolic clearance of BPA via first-pass glucuronide metabolism minimizes internal exposure to free BPA (EFSA 2008). We find this argument to be

simplicistic, ignoring several lines of evidence that internal BPA exposure can be substantial in humans despite rapid conjugation.

The Rapid Metabolism Argument

The EFSA (2008) pointed to pharmacokinetic data in humans showing rapid BPA metabolism to the glucuronide conjugate as reason to decrease emphasis on the low-dose effects seen in rodents. Because only the parent compound binds to the estrogen receptor, conjugation is a detoxification mechanism that represents the major clearance pathway for BPA. Two studies evaluated the metabolic fate of BPA in small numbers of human volunteers ingesting low doses of deuterated BPA (d-BPA), with the labeled chemical used to increase sensitivity and distinguish administered BPA from background sources (e.g., dietary, contamination from plastics in labware) (Völkel et al. 2002, 2008). These studies failed to find detectable concentrations of free d-BPA in human plasma or urine [limit of detection (LOD) = 2.3 $\mu\text{g/L}$ in plasma; see Table 1]. The kinetic profile for the conjugated metabolite (d-BPA glucuronide) showed a rapid peak followed by urinary elimination with a terminal half-life of 5.3 hr (Völkel et al. 2002). These studies have been interpreted as indicating more rapid and complete metabolic clearance of BPA in humans relative to the rat, in which circulating parent compound can be detected and the clearance

of BPA glucuronide is slower ($t_{1/2}$ of 20–80 hr) (EFSA 2008; Völkel et al. 2002, 2008). This was attributed to different elimination pathways in rats versus humans, given that the molecular size cutoff for urinary excretion is larger in humans (550 Da) compared with that in rats (350 Da). This results in elimination of conjugated BPA (404 Da) by biliary/fecal elimination in the rat but via urine in humans (Völkel et al. 2002). This would preclude enterohepatic recirculation in humans, in contrast to rats, in which intestinal β -glucuronidases could break down excreted conjugate and liberate BPA for systemic reabsorption. This rationale has been used to argue the irrelevance of rat data showing effects at low doses, the hypothesis being that at low doses in humans, extensive glucuronidation can essentially prevent fetal exposure to BPA.

With respect to neonatal exposure, the EFSA (2008) recognized that the human data are from adult volunteers and would not necessarily apply to neonates, who might ingest BPA via breast milk or formula. They concluded that early-life immaturity in glucuronidation capacity is likely to be augmented by sulfotransferases given that BPA is a substrate for sulfation, and there is earlier ontogeny of sulfotransferases compared with UDP-glucuronosyltransferases. This was demonstrated for the therapeutic drug acetaminophen, which undergoes a shift in metabolic clearance from primarily sulfation to primarily glucuronidation with increasing postnatal age (Allegaert et al. 2005; Levy et al. 1975; Miller et al. 1976). Therefore, the EFSA (2008) concluded that between sulfation and glucuronidation, sufficient BPA conjugation capacity exists in neonates to prevent exposure to the parent compound.

Based on these arguments, the EFSA maintains the tolerable daily intake (TDI) of BPA for the European Union at 0.05 mg/kg/day (EFSA 2008), an exposure rate that is 200 times greater than that associated with adverse effects in some rodent studies (Vandenberg et al. 2007).

Why Rapid Metabolism Is Not the End of the Story

Although cross-species pharmacokinetic differences exist, there is still ample opportunity

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for human exposure to free BPA. That is because β -glucuronidases exist not only in the intestines but also throughout the body, including the placenta and fetal liver. This creates the potential for local activation of the conjugated form back to free BPA in numerous tissues. This is analogous to the situation for endogenous estrogens, which are transported as sulfate conjugates and can be cleaved to active hormone by tissue sulfatases (Iwamori 2005). β -Glucuronidase activity is widely distributed throughout mammalian tissues and is present in both the endoplasmic reticulum and lysosomes (Paigen 1989; Sperker et al. 1997). Although its primary physiologic role is the degradation of proteoglycans, the enzyme is also able to deconjugate a variety of xenobiotic substrates that have already undergone phase II glucuronidation.

This can result in conjugation–deconjugation cycling that does not involve enterohepatic recirculation, as documented for the drug diflunisal. Treatment of rats with a specific β -glucuronidase inhibitor decreased metabolic clearance of diflunisal by 54% in experiments in which the bile duct was cannulated to prevent enterohepatic recirculation (Brunelle and Verbeeck 1997). Deconjugation of a variety of other xenobiotic metabolites has been documented in human liver preparations, including acetaminophen (Bohnenstengel et al. 1999) and the aromatic amines ben-zidine and 4-aminobiphenyl (Zenser et al. 1999). β -Glucuronidase protein levels and enzyme activity were readily detectable and varied widely in human liver and kidney samples taken from 30 and 18 individuals, respectively (Sperker et al. 1997).

Additional evidence also argues against a simplistic approach. A reanalysis of the National Health and Nutrition Examination Survey (NHANES) BPA urinary biomonitoring results from 1,469 adult participants suggested a longer than expected half-life of BPA (Stahlhut et al. 2009) based on modeling of the fasting time (before sample draw) relative to urinary concentration and the assumption that most BPA exposure comes from the diet. The stable level of urinary BPA may have been due to nondietary BPA exposures, tissue accumulation and storage of free BPA, and/or conjugation–deconjugation cycling of BPA involving β -glucuronidase. It is noteworthy that whereas the terminal half-life is reported to be only 5.3 hr in humans after d-BPA administration (Völkel et al. 2002), the time course shows no additional d-BPA

Table 1. Studies evaluating free BPA in biological fluids.

Study	Exposure	Analytical method	Results	Comments
Domoradzki et al. 2003	Pregnant rats gavaged with ^{14}C BPA, 10 mg/kg, on GD11, GD13, or GD16	Radiochemical HPLC. LOD = 35 $\mu\text{g/L}$; tests run to ensure that procedures did not cleave BPA glucuronide to BPA	Free BPA not detected in many samples early in gestation; GD16 free BPA detectable in all tissues: placenta > maternal plasma > fetus; ratio of free/conjugated: fetus > placenta > maternal plasma	Although fetus had 3.6-fold less free BPA than maternal plasma, free/conjugated BPA ratio was much greater in fetus; may reflect altered conjugation/deconjugation balance in fetal compartment; data from early period before enterohepatic circulation created a secondary C_{max}
Ikezaki et al. 2002	Humans (mother/fetus) background exposure ($n = 32\text{--}38$)	ELISA: details not given, but accuracy checked against standard HPLC method	Free BPA detected in maternal serum, ovarian follicular fluid, and cord blood at similar levels; amniotic fluid was 5 times higher in early but not late pregnancy	Higher free BPA in amniotic fluid early in pregnancy may be related to changing composition: coming from maternal plasma early vs. fetal urine late in gestation; free BPA is in maternal plasma but unlikely in urine
Schönfelder et al. 2002	Humans (mother/fetus) background exposure ($n = 37$)	GC-MS: LOD = 0.91 $\mu\text{g/L}$; LOQ = 0.1 $\mu\text{g/L}$	Free BPA detected in placenta > maternal plasma > fetal plasma; fetal > maternal in 14 of 37 samples, with male fetus > female fetus in these cases	Methods avoided BPA leaching from labware into sample; differences across tissues and sexes suggest free BPA is biologically based rather than from background contamination
Takahashi and Oishi 2000	Pregnant rats dosed on GD18 with 1 g/kg gavage	HPLC: LOD = 5 $\mu\text{g/L}$	Free BPA detected in maternal tissues > fetal tissue \geq maternal blood; fetal $t_{1/2}$ 3 times greater than maternal $t_{1/2}$	High-dose rat study has limited relevance, but it demonstrates distribution to fetal compartment; enterohepatic recirculation likely affects $t_{1/2}$ in both fetus and mother but does not explain longer $t_{1/2}$ in fetus
Takeuchi et al. 2004	Rats background exposure ($n = 10/\text{sex}$)	HPLC: details not clear but appear to involve standard solvent extraction	Free BPA detected in males > females	Sex differential corresponds to lower BPA conjugating capacity in male liver microsomes and lower expression of UGT2B1
Takeuchi and Tsutsumi 2002	Humans: background exposure ($n = 11$ men, 14 women)	ELISA: accuracy checked against HPLC method	Free BPA detected in males > females	Free BPA correlated with serum testosterone in both men and women, suggesting androgen effect on BPA fate
Tan and Mohd 2003	Humans: background exposure ($n = 180$ females)	GC-MS: LOD = 0.05 $\mu\text{g/L}$	Free BPA detected in cord blood in 88% of samples	Demonstrated potential utility of biomonitoring free BPA and other alkylphenols in cord blood
Völkel et al. 2002	Humans: dosed orally with 5 mg d-BPA ($n = 4$)	LC-MS: LOD _{plasma} = 2.3 $\mu\text{g/L}$; LOD _{urine} = 1.4 $\mu\text{g/L}$	No detection of free BPA even though d-BPA glucuronide was high (160 $\mu\text{g/L}$ blood); d-BPA glucuronide, $t_{1/2} = 5.3$ hr	Lack of free BPA attributed to "practically complete" first-pass metabolism and lack of enterohepatic circulation; detection of free BPA in background samples attributed to plastic contamination
Völkel et al. 2008	Humans: background exposure ($n = 287$)	LC-MS: LOD _{urine} = 0.3 $\mu\text{g/L}$; LOQ _{urine} = 1.25–5 $\mu\text{g/L}$	10% of urine samples with low detection ($< \text{LOD}$, $< \text{LOQ}$)	Free BPA in urine attributed to contamination from house dust or plastics because BPA was also found in blanks; d-BPA dosing did not find free BPA in urine
Völkel et al. 2008	Human: dosed orally with 5 mg d-BPA ($n = 1$)	HPLC-MS: LOD _{urine} for d-BPA was not stated	Free d-BPA not detected in any urine samples from this subject	Results used to assert that if free BPA is detected in urine, it is from contaminants and not free BPA
Yamada et al. 2002	Humans: background exposure ($n = 200$ women)	ELISA: LOD = 0.2 $\mu\text{g/L}$; method previously validated against HPLC method	Second trimester serum samples in Japanese women showed steady decline in free BPA from 1989 to 1998	No clear explanation of decreasing BPA in serum, but results were significant ($p < 0.001$); free BPA lower in amniotic fluid than maternal serum

Abbreviations: C_{max} , maximum concentration; ELISA, enzyme-linked immunosorbent assay; GC, gas chromatography; GD, gestation day; HPLC, high-performance liquid chromatography; LC, liquid chromatography; LOQ, limit of quantification; MS, mass spectrometry.

glucuronide removal beyond 20 hr (additional data from Völkel et al. 2002 presented in Teeguarden et al. 2005). This is consistent with delayed excretion due to long-term tissue storage and/or conjugation–deconjugation cycling. The terminal half-life in cynomolgus monkeys dosed orally with ^{14}C -BPA was 9.7 hr (Kurebayashi et al. 2002). Thus, the cross-species difference in terminal half-life of BPA glucuronide may not be as large as stated (EFSA 2008). Finally, there are uncertainties when comparing half-life across studies involving administered oral doses that were 100–1,000 times higher in the rat study (Domoradzki et al. 2003; Pottenger et al. 2000) than in the human study (Völkel et al. 2002). Although glucuronidation is considered a high-capacity pathway, one cannot rule out the possibility that the rat to human difference in half-life was affected by the difference in administered dose.

Developmental studies suggest rapid ontogeny of β -glucuronidase, because it is detected prenatally in liver, kidney, and lung in a variety of laboratory species, with activity particularly high in placenta (Lucier et al. 1977). Human placenta also has considerable β -glucuronidase activity, and this enzyme is critical for proper *in utero* development (Collier et al. 2009; Paigen 1989; Sperker et al. 1997). An inherited deficiency leads to hydrops fetalis, a birth defect related to improper fetal breakdown of mucopolysaccharides and water accumulation. Because glucuronidation capacity is immature in early life, the net balance tends to be toward deconjugation in the animal models and tissues studied (Lucier et al. 1977). This makes BPA deconjugation a potentially

important pharmacokinetic factor during the perinatal period.

Thus, it is apparent that one has to consider the potential for β -glucuronidase-mediated deconjugation of BPA glucuronide in placental and fetal tissues. Even though glucuronidation may be rapid, the reported terminal half-life of circulating BPA glucuronide, 5.3 hr, affords ample opportunity for transport to placenta and deconjugation back to BPA. This and the fact that the fetus itself contains β -glucuronidase increase the chances for substantive fetal exposure to free BPA. By focusing on rapid conjugation and not considering sites of deconjugation other than the intestine (enterohepatic recirculation), the EFSA has not considered the implications of BPA glucuronide deconjugation in placental and fetal tissues. This issue may be a key determinant of cross-species extrapolation of BPA internal dose, but it has yet to be addressed in BPA pharmacokinetic models (Edginton and Ritter 2009; Teeguarden et al. 2005) or regulatory determinations.

What Is the Meaning of Free BPA Detection in Human Samples?

The Völkel et al. (2002, 2008) human dosing studies failed to detect free BPA (Table 1) and so were interpreted by the EFSA (2008) as supportive of rapid metabolic clearance and negligible exposure in adults as well as the fetus. However, numerous other studies have detected free BPA in both humans and rats, either associated with general background exposure or in experiments where rodents were dosed with BPA (Table 1). Free BPA

was detected by a variety of methods and was found not only in adult blood but also in placental and fetal samples. Dekant and Volkel (2008) argued that the detection of free BPA in such studies may result from background contamination from labware and indoor dust. Further, some free BPA may be formed by cleavage from the glucuronide present in the sample when readying the sample for analysis (Dekant and Volkel 2008). However, many of the cited studies were aware of these potential artifacts and took steps to prevent false-positive detection of free BPA. Further, our review of the free BPA data derived from these studies suggests that this result is not artifactual. Quite the contrary, these data suggest patterns of occurrence that have important implications for BPA risk assessment.

Figure 1 includes studies in which free BPA was detected in rats dosed during pregnancy, demonstrating maternal serum BPA greater than fetal BPA in two studies (Domoradzki et al. 2003; Takahashi and Oishi 2000). In a study in which placental BPA was also measured, the placenta had a higher BPA concentration than did the maternal or fetal compartments. This is consistent with human free BPA data collected in a biomonitoring study of pregnant women exposed to background sources of BPA (Schönfelder et al. 2002). Once again, placenta had the highest concentration, followed by maternal and fetal compartments (Figure 2). These trends have a plausible biological basis in that placenta has extensive β -glucuronidase activity (see above) and so may be an important site of metabolite deconjugation and resultant fetal exposure. Although peak fetal concentrations of free BPA were less than maternal concentrations in both rats and humans, a more detailed time course in rats indicated that cumulative free BPA exposure was actually greatest in the fetus [fetal area under the curve was 73% greater than maternal (Takahashi and Oishi 2000)]. This may reflect ongoing deconjugation in placenta and fetus that prevents free BPA from declining as rapidly as in the maternal system. Also of note in this study was the finding of much higher concentrations of free BPA in liver and kidney compared with blood, again suggesting the importance of local tissue deconjugation and/or binding in determining free BPA dose. In this study, Takahashi and Oishi (2000) used a very large dose (1 g/kg), so their results, although consistent with the others cited, should be repeated at more relevant doses.

Additional evidence of BPA deconjugation during gestation comes from rat data showing that the ratio of glucuronide to free BPA varies across maternal, placental, and fetal compartments (Figure 1). Although Domoradzki et al. (2003) reported no selective tissue affinity for BPA or its metabolite, their data show a continuous decrease in glucuronide to free

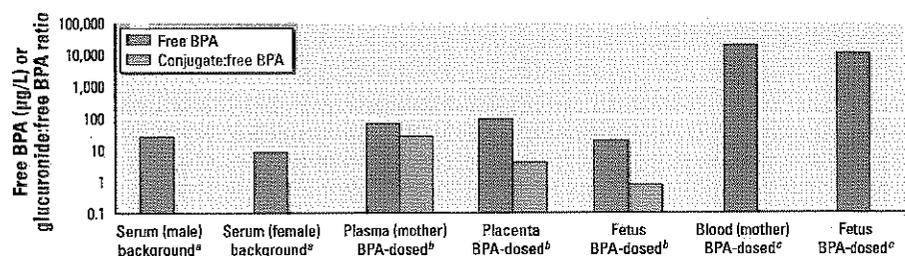


Figure 1. Free BPA and ratio of conjugate to free BPA in rats from background exposure or after BPA dosing. ^aData from Takeuchi et al. (2004). ^bData from Domoradzki et al. (2003). ^cData from Takahashi and Oishi (2000).

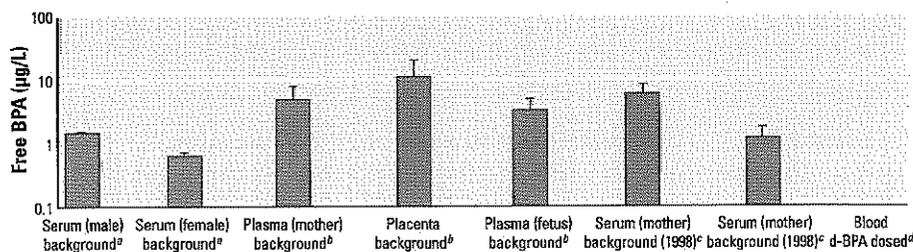


Figure 2. Free BPA in humans from background exposure or after BPA dosing. ^aData from Takeuchi et al. (2002). ^bData from Schönfelder et al. (2002). ^cData from Yamada et al. (2002). ^dData from Völkel et al. (2002); LOD = 2.3 µg/L.

BPA ratio across these compartments, suggesting a greater role for deconjugation in the placenta and fetus than in maternal blood.

The biological plausibility of the free BPA data is also supported by the difference between sexes in both rats and humans (Figures 1 and 2). Male rats exhibited greater free BPA than did females, consistent with decreased expression of the main BPA-glucuronidating enzyme [UDP-glucuronosyltransferase 2B1 (UGT2B1)] in male rat liver (Takeuchi et al. 2004). The fact that this was also seen in humans (Figure 2) suggests that BPA metabolic fate is under hormonal control in both species. The sex differential appears to exist very early in human development, because free BPA was greater in male than female fetuses of women receiving BPA exposure from background sources (Schönfelder et al. 2002).

The risk implications of free BPA detections need to be explored based upon dose-response assessment and suitable physiologically based pharmacokinetic (PBPK) modeling that can relate internal dose of free BPA to adverse effect. The existing PBPK models (Edginton and Ritter 2009; Teeguarden et al. 2005) have not considered the influence of local deconjugation reactions. In the only attempt to simulate free BPA concentrations, Teeguarden et al. (2005) were not able to reproduce the free BPA results for rat plasma at the later time points (4 and 8 hr after dosing) even though their model included enterohepatic recirculation and plasma protein binding. There is a clear need to improve modeling efforts with respect to free BPA in maternal, fetal, and neonatal tissues across species, with metabolite deconjugation a potentially important element. Better calibration of the models against the database of free BPA detection (Table 1) should be part of this effort. This may be facilitated by *in vitro* studies that evaluate the conjugation-deconjugation activity of placenta and other human and rodent tissues. Such *in vitro* data along with additional human volunteer studies evaluating BPA concentration after controlled exposures (e.g., Carwile et al. 2009) will inform the degree of variability in human BPA pharmacokinetics. This is particularly uncertain given the small numbers of adult subjects that were involved in the detailed pharmacokinetic studies thus far reported (Völkel et al. 2002, 2008; Table 1). A PBPK model parameterized with empirical conjugation and deconjugation rate constants and calibrated for free BPA is needed to relate the dose response for toxic effects found in rodents to humans.

What about BPA in Neonates?

The EFSA also considers dietary BPA exposures in neonates to be insignificant because of rapid metabolism, in this case not because of glucuronidation but because of sulfation.

Based on analogy with acetaminophen, BPA may be conjugated with sulfate rather than glucuronide in neonates because of the earlier development of sulfotransferases. However, sulfation does not end the biological activity of endogenous hormones, so there is no reason to believe it will do so for sulfated BPA. Sulfated estrogens, mainly in the form of estrone sulfate, have a long half-life in blood, where their concentration is much higher than the active hormone (Nakamura et al. 2005). These conjugates act as a circulating reservoir of inactive hormone that can be deconjugated in local tissues by arylsulfatase C, a widely expressed microsomal enzyme that is especially prevalent in estrogen-responsive tissues (Reed et al. 2005; Tobacman et al. 2002). Given that sulfotransferases also exist in these tissues, the balance between conjugation and deconjugation at a particular life stage and in a specific tissue is a key determinant of local estrogen dose. This has not been studied for BPA and is a critical data need for developing improved PBPK models for the postnatal period. However, it is reasonable to assume that the BPA sulfate conjugate would be subject to deconjugation in a manner similar to endogenous sulfated estrogens. This is pertinent to the postnatal period as arylsulfatase C activity develops *in utero* and is readily detectable in human neonatal liver (Richard et al. 2001). Thus, sulfation of BPA in neonates does not guarantee negligible internal dose as assumed by the EFSA.

Another consideration is that this argument is based on analogy with acetaminophen. However, it is uncertain whether the sulfotransferases present in neonates will be as efficient in conjugating BPA as they are for acetaminophen. This represents another key data gap. Finally, genetic polymorphism in major sulfotransferases such as *SULT1A1*2* can decrease conjugating activity 2- to 10-fold (Hildebrandt et al. 2007; Nagar et al. 2006; Ohtake et al. 2006). This can be an important source of interindividual variability in neonatal BPA conjugation that is not considered in the EFSA assessment.

Summary

Free BPA concentrations have been detected in a wide range of both human and rodent studies and likely reflect the *in vivo* condition rather than artifact. This provides evidence of exposure to free BPA in human adults and fetuses despite rapid first-pass glucuronidation. Deconjugation at local tissue sites by the action of β -glucuronidase and arylsulfatase C provides a plausible mechanism. The EFSA's review of the pharmacokinetic evidence concludes that cross-species differences in BPA glucuronide fate (enterohepatic recirculation in rats; urinary excretion in humans) makes low-dose studies in rodents less relevant for human risk assessment

(EFSA 2008). The EFSA also believes that on the basis of rapid BPA metabolism and excretion in humans, fetal and neonatal exposure is negligible. However, detection of free BPA in human and rodent placenta and fetus is contrary to that opinion and argues for placing importance on deconjugation reactions in future risk assessments.

The EFSA TDI of 0.05 mg/kg/day (EFSA 2008) is orders of magnitude greater than the dose found to produce effects in some rodent studies. Pharmacokinetic differences across species would have to be enormous to justify acceptance of a TDI that far above possible effect levels. The points raised above demonstrate the uncertainty in the pharmacokinetic argument for maintaining the current EFSA TDI. Efforts should be placed on deciphering the dose-response relationship in rodent studies and applying it to human risk assessment based upon PBPK models that account for metabolite deconjugation in conjunction with other pharmacokinetic factors. Such PBPK models do not currently exist, and the existing models (Edginton and Ritter 2009; Teeguarden et al. 2005) cannot fully simulate the human and rodent data. Therefore, as an interim measure, one may choose to directly apply the rodent dose-response relationship to humans and seek additional mechanistic or epidemiologic data to refine the human risk assessment.

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Daily Bisphenol A Excretion and Associations with Sex Hormone Concentrations: Results from the InCHIANTI Adult Population Study

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Daily Bisphenol A Excretion and Associations with Sex Hormone Concentrations: Results from the InCHIANTI Adult Population Study

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Short running head

Bisphenol A Daily Excretion and Sex Hormones

Abbreviations

BMI	Body Mass Index (BMI)
BPA	Bisphenol A
CV	coefficients of variation
HESI	heated electrospray ionisation interface (for MS/MS)
HPLC	high performance liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LOD	limit of detection
LOQ	limit of quatitation
MS/MS	tandem mass spectrometry
RIA	radioimmunological assay (RIA)
RSD	relative standard deviations
SHBG	Sex Hormone Binding Globulin
SPE	solid-phase extraction (SPE)
S/N	ratio of the signal height (S) to the noise height (N).
UER	Urinary Excretion Rate

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UGT uridine diphosphate-glucuronosyl transferase
NHANES US National Health and Nutrition Survey

Key words

endocrine disruption, androgen, anti-androgen, bisphenol A, human biomonitoring, health effects, InCHIANTI

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Conflicts of interest:

CM and PM are both employed by Brixham Environmental Laboratory, AstraZeneca UK Ltd, but their input was limited to conducting and documenting the BPA assays, blind to the other data examined. The analysis of BPA samples on contract was funded from independent Peninsula College of Medicine and Dentistry sources. The authors declare that they have no other actual or potential competing financial interests.

Abstract

Background: Bisphenol A is a high production volume chemical widely used in food and drinks packaging. Numerous studies demonstrate that BPA can alter endocrine function in animals, yet human studies remain limited.

Objective: To estimate daily excretion of BPA in adults and to examine hypothesized associations with serum estrogen and testosterone concentrations.

Design, setting and participants: Cross-sectional analysis of associations in the InCHIANTI study, an Italian population sample. Included were 715 adults aged 20 through 74 years. BPA concentrations were measured by liquid chromatography mass spectrometry (LC-MS) in 24 hour urine samples.

Main outcome measures: serum concentrations of total testosterone and 17 beta-estradiol.

Results: Geometric mean urinary BPA concentration was 3.59 ng/ml (95% CI 3.42 to 3.77), and mean excretion was 5.63 $\mu\text{g}/\text{day}$ (5th population percentile 2.1 $\mu\text{g}/\text{day}$, 95th percentile 16.4 $\mu\text{g}/\text{day}$). Higher excretion was found in men, younger respondents and with increasing waist circumference ($p=0.013$) and weight ($p=0.003$).

Higher daily BPA excretion was associated with higher total testosterone concentrations in men, in age and study site adjusted models ($p=0.044$) and in models adjusted additionally for smoking, measures of obesity and urinary creatinine concentrations ($\beta=0.046$ CI 0.015 to 0.076, $p=0.004$). There were no associations with the other serum measures. There were no associations with the primary outcomes in women, but there

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was an association between BPA and SHBG concentrations in the 60 pre-menopausal women.

Conclusion: Higher BPA exposure may be associated with endocrine changes in men.

The mechanisms involved in the observed cross-sectional association with total testosterone concentrations need to be clarified.

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Introduction

Bisphenol A (BPA) is a synthetic compound that is suspected to act as an endocrine disruptor, i.e. a compound capable of causing dysfunction to hormonally regulated body systems (Talsness et al. 2009). BPA is used as a monomer in polycarbonate plastics and in the epoxy resins that are used to line food and beverage containers and is one of the world's highest production volume chemicals (Burrige 2003). Widespread and continuous daily exposure to BPA is believed to occur primarily through the diet (Stalhut et al. 2009) but also through drinking water, dental sealants, from dermal exposure and inhalation of household dusts. The presence of measurable concentrations of metabolites has been reported in the urine of > 90% of people in population representative samples from across the globe (Calafat et al. 2008, Vandenberg 2007).

Most studies of the health effects of BPA have focused on its well-documented estrogenic activity, with reports of both estrogen agonist (Lee et al. 2003) and androgen antagonist activity (Bonefeld-Jorgensen et al. 2007, Lee et al. 2003, Okada et al. 2008). Suppression of aromatase activity has been observed in laboratory studies (Bonefeld-Jorgensen et al. 2007), as has binding to alternative nuclear receptors including the aryl hydrocarbon receptor (AhR) (Kruger et al. 2008) and estrogen-related receptor γ , the function of which remains unknown (Okada et al. 2008). In addition, BPA has been reported to cause thyroid hormone disruption (Moriyama et al. 2002), altered pancreatic beta-cell function (Ropero et al. 2008) and obesity-promoting effects (Newbold et al. 2008). The potential for low dose effects has prompted debate on the requirement for revision of current legislation on recommended safe daily exposure levels (Beronius et al. 2010, vom Saal et al. 2007)

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Based on the animal and laboratory evidence, we previously hypothesized that higher urinary BPA concentrations would be associated with adverse human health effects.

Using data from the US National Health and Nutrition Survey (NHANES) 2003/04, the first large-scale population representative epidemiological data on urinary BPA concentrations with sufficient power to detect low-dose effects, we showed for the first time a clear correlation between BPA exposure and disease in humans (Lang et al. 2008). Higher BPA concentrations in NHANES respondents were associated with diagnoses of cardiovascular disease and diabetes but not with other common diseases, suggesting specificity of the reported findings (Melzer et al. 2008, 2009). We recently provided independent replication of the association with cardiovascular disease in an entirely new study sample, provided by the 2005/6 NHANES survey (Melzer et al. 2010), making chance an implausible explanation for our results.

Studies to clarify the mechanisms of these associations are clearly a priority. A substantive literature documents the disruption of circulating reproductive hormone concentrations following BPA exposures in animal models (reviewed in Richter et al. 2007; see also Bonefeld-Jorgensen et al. 2007, Goodman et al. 2008, Talsness et al. 2009). Studies of human populations have until now been limited to very small sample sizes. A significant, positive relationship was reported between circulating androgen concentrations and BPA exposure in a small study of 26 normal women and 47 with ovarian dysfunction (Takeuchi et al. 2004). More recently, Meeker et al. (2010) studied serum thyroid and reproductive hormone levels in 167 men recruited through an

infertility clinic and observed inverse relationships between urinary BPA concentrations and free androgen index (ratio of testosterone to sex hormone binding globulin), estradiol and thyroid stimulating hormone. Given these findings, we hypothesized that higher urinary BPA concentrations would be associated with altered reproductive hormone concentrations in serum. Since a limitation of previous studies has been their reliance on single spot urine samples, we based our current analysis on 24 hour urine collections, to provide a direct measure of daily excretion rates. The study population was selected from the InCHIANTI study, a population representative sample based in Chianti, Italy, allowing for a first report of daily BPA excretion levels in a large European cohort.

Materials and Methods

Study population

The InCHIANTI study (<http://www.inchiantistudy.net/study.html>) was designed to identify risk factors for mid and late-life morbidity and has been described extensively elsewhere (Ferrucci et al. 2000).

Briefly, InCHIANTI is a prospective population-based study covering a suburban and rural town population. Participants were selected from adults living in Greve in Chianti and Bagno a Ripoli, Tuscany, Italy, using a multistage sampling method. N=296 adults aged <65, n= 533 aged 65 to 74 and n=102 aged 75 and over (response rate of 91.6% from baseline interview) were randomly selected from the population using city registries. In line with previous work we have limited our analysis here to those aged

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≤74. The Istituto Nazionale Riposo e Cura Anziani Institutional Review Board provided ethical approval for the study. Participants gave informed consent to participate, or if they were unable a close relative provided surrogate consent.

Analysis of urinary BPA concentrations

Analysis of samples was performed (under contract) at the Brixham Environmental Laboratory in compliance with Good Laboratory Practice, EU Directive 88/32/EEC. Because orally administered BPA is considered to be rapidly and completely excreted, urine is the body fluid most appropriate for biomonitoring assessment of BPA exposure, as described by Calafat et al. (2005). We measured total (free and conjugated) urinary concentrations of BPA based on the methods employed by NHANES (Calafat et al. 2008) and adopted by the Division of Environmental Health Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, (CDC) i.e. sample preparation and on-line solid-phase extraction (SPE) coupled with high performance liquid chromatography (HPLC)-isotope dilution tandem mass spectrometry (MS/MS) with peak focusing.

Analyses were carried out using a commercially available, integrated on-line SPE-LC system (SymbiosisTM Pharma system, Spark Holland BV, Emmen, NL) coupled with a triple quadrupole mass spectrometer equipped with a heated electrospray ionisation (HESI) interface HESI-MS/MS (TSQ Quantum Ultra AM, Thermo Scientific, Hemel Hempstead, UK). Two major advantages of the SymbiosisTM Pharma system are that; a new SPE cartridge is used for every analysis and one SPE cartridge is prepared whilst one is being analyzed. This enabled a 7 minute SPE-LC-HESI/MS/MS run time for each

analysis point. A linear calibration was obtained from 0.50 to 100 $\mu\text{g L}^{-1}$ ($R^2 > 0.996$). The limit of detection (LOD) was $< 0.50 \mu\text{g L}^{-1}$ BPA, the limit of quantitation (LOQ) was $0.50 \mu\text{g L}^{-1}$ BPA (the lowest calibration standard with a ratio of the signal height (S) to the noise height (N). $S/N > 10$ and relative standard deviations (RSD) $< \pm 20\%$, all other standards $< \pm 15\%$).

Outcomes

Participants who consented to donate a blood sample were asked also to collect the urine produced in 24 hours (24h) into a collection vessel containing 3g of boric acid, as preservative. Over the 3 days prior to blood and urine collection, the subjects consumed a diet free of meat and fish. On the morning of the day before the blood samples was drawn, participants urinated and flushed away the first voided urine, then began the urine collection. During the day and night all the produced urine was saved into the plastic bottle stored at room temperature or in the refrigerator. After 24h, bottles were weighed and the total volume measured in the clinic.

First thing the next morning, after having been sedentary for 15 minute, the participants fasting blood samples were collected for routine blood examination. Aliquots of serum and plasma were subsequently prepared and stored at -80°C for additional analyses. A 24h urine sample aliquot (70 mL) was stored at -20°C until further analyses.

Testosterone circulates in the blood bound predominantly to protein, with approximately 40% bound to the high affinity sex hormone binding globulin (SHBG) and 60% to albumin with lower affinity. Measurement of serum testosterone typically includes

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estimation of total testosterone (free plus bound), free testosterone (non protein bound) and bioavailable testosterone (non SHBG bound).

Total testosterone was assessed through a commercial radioimmunological assay (RIA) kit (Active Testosterone RIA DSL-4000; Diagnostic Systems Laboratories, Webster, TX, USA, distributed by Chematil, s.r.l., Angri (SA), Italy). The minimum detection limit was 0.08 ng/mL. Intra-assay coefficients of variation (CVs) for 3 different concentrations ranged from 7.8%-9.6% whilst inter-assay CVs ranged from 8.4%- 9.1%. Results were transformed and reported as ng/ml according to the manufacturer's instructions.

Sex hormone binding globulin (SHBG) level was measured by radioimmunoassay (IRMA DSL-7400; Diagnostic Products Corporation, Los Angeles, CA, USA). The analytical sensitivity was 3 nmol/L. The intra-assay CVs for 3 different concentrations were from 1.1% -3.7% and inter-assay CVs were from 8.7 -11.5%.

Free testosterone was estimated from measured total testosterone, SHBG and albumin (4.3 g/dl) using the method of Vermeulen A et al. (1999), see the International Society for the study of the Aging Male website at <http://www.issam.ch/freetesto.htm> for a worked example.

Estradiol levels were measured using an ultra-sensitive radioimmunological assay (RIA) (Ultra-sensitive Estradiol RIA DSL-4800; Diagnostic Systems Laboratories, Webster, TX, USA, distributed by Chematil, s.r.l., Angri (SA), Italy). The theoretical sensitivity was 2.2 pg/mL. Intra-assay CVs across 4 different concentrations ranged from 6.5% - 8.9% whilst inter-assay CVs ranged from 7.5% to 12.2% (at 108.7 pg/mL).

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Statistical analysis

Descriptive statistics of urinary BPA concentration and serum hormone levels were tabulated. We calculated geometric means and distribution percentiles of two different BPA measures. First of all, we measured the BPA volume-concentration (expressed as microgram of BPA per litre of urine). Then, we multiplied the BPA concentration by the urine collection rate (L/day), measured considering the urine volume collected in 24 hours, and, hence, we obtained the Urinary Excretion Rate (UER) of BPA (expressed as micrograms per day).

We performed multivariate linear regression model to study the association between BPA and a broad range of demographic covariates and possible confounders. Because the concentrations of daily BPA excretion was not normally distributed, we used natural log transformation when BPA was considered the dependant variable. BPA values were not transformed when it was considered as explanatory variable in serum hormone examination. In all analyses, an upper age cut-off was chosen at 75 years to minimize problem of co-morbidity.

We adjusted our models with selected different covariates. Variables included were age, reported in years at the last birthday and was used as continuous variable; the two municipalities (study sites) where participants live; waist circumference (cm) and weight (kg); Body Mass Index (BMI) was calculated as the weight (kg) divided by the square of height (metre, m). BMI was tested as continuous variable and as categorized dummy

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variable: subjects were divided in underweight (<18.5), recommended weight (18.5-24.9), overweight (25.0-29.9), obese I (30.0-34.9), or obese II (≥ 35). We also considered the smoking status which appeared correlated to BPA in unadjusted models.

Urinary creatinine concentration is commonly used to adjust within-day variation in metabolite analysis from single spot urine sample (Barr et al. 2005). Linear regression analysis using the outcome (hormone) measures as the dependant variable was performed first considering all the subjects and then male and female separately. Data analysis was performed using STATA 10 SE (StataCorp, College Station, Texas); $p < .05$ was considered significant.

Results

The geometric mean urinary concentration of BPA was 3.59 ng/ml (95% CI 3.42 to 3.77) (Table 1). Based on the 24 hour urine collection, the daily excretion rate of BPA had a geometric mean of 5.63 $\mu\text{g}/\text{day}$ but varied widely. The distribution was skewed, with a 10th percentile of 2.6 $\mu\text{g}/\text{day}$ (95%CI 2.5 to 2.8) and a 90th percentile of 11.8 $\mu\text{g}/\text{day}$ (CI 10.9 to 12.7). Daily BPA excretion was lower in women than in men ($p < 0.001$ in age, sex and study site adjusted models) and lower with advancing age ($p < 0.001$). Identical results were obtained both with and without correction for creatinine. In age, sex and study site adjusted models (table 2), no associations were found between daily BPA excretion and years of education or smoking status. Associations were found with waist circumference

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($\beta=0.0062$ CI 0.0016 - .0108, $p=0.013$) and with weight ($\beta=0.0064$ CI 0.0023 - 0.0104, $p=0.003$).

In age and study site adjusted models for men, there was no association between BPA excretion and 17 beta-estradiol. However, there was a significant association between daily BPA excretion and total testosterone concentration (coefficient = 0.0237 CI 0.0006 to 0.0468, $p=0.044$). In models adjusted for age, study site, smoking, BMI, weight, waist, and urinary creatinine, the BPA association with total testosterone levels was highly significant ($\beta=0.046$ CI 0.015 to 0.076, $p=0.004$) (Table 3).

To explore the association with testosterone in men further, we examined associations with the derived measure of free testosterone, based on Sex Hormone Binding Globulin (SHBG) concentrations. The association between BPA excretion and free testosterone narrowly missed significance ($p=0.075$ in fully adjusted models).

In women, the geometric mean 17 beta-estradiol concentration was 6.89 pg/ml (table 4), but this varied dramatically by menopause status: 22.4 pg/ml (CI 16.7 to 30.0) in the 57 pre-menopausal women and 5.3 pg/ml (4.8 to 5.07) in the 290 post-menopausal women. In models testing hormone associations with BPA excretion in women (table 4), we found no significant associations for either estradiol or total testosterone. Both sex hormone binding globulin (SHBG) concentration and the derived measure of free testosterone showed significant associations with BPA excretion in premenopausal women, although it should be noted that the method used (direct measure of free

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testosterone by radioimmunoassay and calculation of free androgen index) is not designed for measuring androgen concentrations in women, where the concentrations involved are at the very lowest limits of detection (Vermeulen et al. 1999, Miller et al. 2004).

Sensitivity analysis

For a sensitivity analysis of our main finding, we examined the relationship between daily BPA excretion and total testosterone levels in men, excluding outlier BPA values above 25 µg per day (n=7 removed ranging from 25.29 to 41.12 ug/day) (see Figure 1 for unadjusted model). In fully adjusted models as above, BPA excretion per day remained associated with Total Testosterone concentrations in men ($\beta=0.0521$, CI 0.0172 to 0.08703, $p=0.004$).

Post-hoc analyses for bio-available testosterone showed similar patterns to those reported for free testosterone (data not shown). Associations with estradiol - testosterone ratios were non-significant.

Discussion

In this study, we report for the first time the daily excretion levels of BPA in European adults in a large scale and high quality population representative sample. After adjusting for potential confounders, we showed that higher BPA daily excretion was associated with an increase in serum total testosterone concentration in men.

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These results are important because they provide a first report in a large-scale human population of associations between elevated exposure to BPA and alterations in circulating hormone levels. They also illustrate that the extent of exposure to BPA is similar in this European mixed urban and rural population to exposures seen in the general adult population of the USA (Calafat et al. 2008). Previous studies of the relationship between human exposure to BPA and endocrine function are sparse and involve reported alterations in androgens (gonadotrophins or testosterone) in urine or serum in both male and female subjects, although the numbers of participants were small (Hanaoka et al. 2002, Takeuchi and Tsutsumi, 2002, Takeuchi et al. 2004). Hanaoka (Hanaoka et al. 2002) studied 42 occupationally exposed male production workers and age matched controls and showed that urinary BPA concentrations were inversely associated with FSH, but not with free testosterone or LH. In a later study of 167 men recruited through an infertility clinic, BPA concentrations in urine were positively associated with both FSH and FSH: inhibin ratio and inversely associated with estradiol: testosterone ratio. Since FSH and inhibin B are the two hormones considered most predictive of semen quality, the authors concluded that BPA may have been associated with adverse effects on Sertoli cells or their FSH receptors, leading to altered inhibin B production and reduced semen quality. In a recent study, rats exposed to BPA *in utero* did not show significant changes in circulating testosterone levels in adulthood, suggesting normal functioning of Leydig and Sertoli cells (Thuillier et al. 2009). Since estrogens and androgens can exert differential effects in function depending on the cell type and its stage of development, the consequences of BPA exposure on adult reproductive and somatic tissues merits further attention (Goodman et al. 2008)

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Our results showed an association with total testosterone concentrations but no significant trend in 17 beta estradiol levels with higher BPA excretion in men. The results reported by Meeker et al. (2010) are consistent with those reported here, although the positive trend ($p=0.17$) between BPA and testosterone reported by Meeker et al. (2010) did not reach statistical significance in their smaller study. Mendiola et al. (2010) recently reported finding no association between urinary BPA concentrations and testosterone levels in 375 male partners of pregnant women: in addition to differences in study group, their urinary BPA concentrations appear substantially lower than in our study sample.

Plausible explanations for our finding of an increase in total testosterone include a reduction in aromatase activity (Arkingbemi et al. 2004, Huang and Leung, 2009, Nativelle-Serpentini et al. 2003), which would lead to a decrease in the conversion of testosterone to estradiol. Since BPA has been shown to possess anti-androgenic activity (Bonefeld-Jorgensen et al. 2007, Lee et al. 2003), an alternative explanation could be that blockade of androgen binding sites alters feedback control mechanisms, leading to an increase in circulating testosterone. Lee et al (2003) showed BPA to affect multiple steps in the activation and function of the androgen receptor, including non-competitive inhibition of binding of endogenous androgens, nuclear localization and transactivation, with uncertain consequences for androgen homeostasis. Associations with the derived measure of free testosterone narrowly missed statistical significance.

Alternatively, there could be differential effects of BPA on the metabolism of testosterone and estrogen. A study of steroid hormone production in rat ovarian cells

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showed that BPA increased both testosterone synthesis and the mRNA expression of steroidogenic enzymes (Zhou et al. 2008). BPA also significantly decreased the activity of enzymes involved in the hydroxylation of testosterone, including the cytochrome P450 isoforms testosterone 2 α -hydroxylase and testosterone 6 β -hydroxylase, CYP2C11/6 and CYP3A2/1 in isolated rat livers (Hanioka et al. 1998), both of which could lead to a net increase in circulating testosterone. The possibility that BPA could interfere with the radioimmunoassay used to quantify serum testosterone is unlikely given the low cross-reactivity shown by the anti-testosterone antibody used in the assay and is further discounted by mathematical modelling studies showing negligible effects of xenoestrogens on the displacement of bound hormone and tracer during binding and extraction steps *in vitro* (Heringa et al. 2004).

It is also plausible that an androgenic environment leads to alterations in the metabolism of BPA, i.e. reverse causation. Metabolism of BPA in the intestine and liver catalyzed by uridine diphosphate-glucuronosyl transferase (UGT) yields the major urinary metabolite BPA-glucuronide (Teegarden et al. 2005). The level of both UGT activity and transcription has been shown to be down-regulated by androgens (Guillemette et al. 1997, Takeuchi et al. 2004), which could result in an increase in serum BPA concentration under hyperandrogenic conditions. However, it is unlikely that such metabolic change could alter 24 hour urinary BPA excretion in the context of the population level repeated ingestion of BPA and the limited increase in testosterone concentrations evident in our analysis.

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Our results provide a first report of the extent of human exposure to BPA in a large-scale European population. Urinary BPA concentrations have previously been reported in 100 pregnant European females, with 82% of the study population showing detectable levels of BPA, median concentration 1.2ng/ml (Ye et al. 2008). This is lower than the mean value here 3.59 ng/ml (95% CI 3.42 to 3.77)) although there are differences in age and gender profiles. Most studies have reported values from spot urine samples with or without correction for creatinine, with mean concentrations around 3ng/ml (Dekant and Volkel, 2008, Vandenberg et al. 2010), and 95th percentiles in the range 11.5 ng/ml (Ye et al. 2005) to 16 ng/ml (Calafat et al. 2005). Here, we used 24h urines to calculate a mean daily excretion rate of 5.63µg/day (95% CI 5.67-5.90µg/day). Median daily urinary excretion of BPA of 1.3-5µg/day were reported in an earlier Japanese study, with a maximum daily intake of BPA per body weight of 0.23 µg/kg/day based on 24h urine samples collected from 36 men (Arakawa et al. 2004). The median daily uptake was given as 0.02 µg/kg body weight. In controlled, acute human exposure studies, peak urinary concentrations of BPA metabolites were 4500-6800 µg/l 6 h after oral administration of 60-80µg/kg body weight. Based on these figure and assuming complete and rapid excretion, Dekant and Volkel (2008) suggest that a daily excretion rate of around 5µg/l, as seen in the general population, indicates ingestion of less than 25µg of BPA in the hours prior to sampling, (the maximum daily reference dose is 50µg/kg/day). However, there are no actual *in vivo* data on the rate at which unconjugated BPA is converted to BPA-glucuronide in humans, only estimates. BPA is lipophilic with a log of the octanol-water partition co-efficient (K_{ow}) of between 2.2-3.82 and it may partition to lipid rich tissues, a suggestion supported by population-based half lives for BPA

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calculated by Stahlhut et al. (2009) to be significantly longer than previous predictions of 6h. Given the correlations with BMI and waist circumference seen here, a true estimation of exposure rates remains a priority.

There are limitations to this study that should be borne in mind when interpreting the results. In the first instance, replication is required in an independent study population to exclude chance as an explanation, although the small p-value in fully adjusted models and the broad consistency with previous work suggest this is unlikely.

The analysis is based on a single day of BPA excretion, which is clearly not a perfect measure of longer term exposure given that human health effects are most likely associated with long-term low-dose exposure. However, using the 24 hour urine specimens is likely to be more accurate than previously published work, which has been based on a spot urine sample with post hoc adjustment to try to correct for concentration effects. Spot urine samples themselves have been shown to be moderately sensitivity for predicting an individual's tertile categorization. (Mahalingaiah et al. 2008).

Misclassification due to this single day snapshot of excretion will have resulted in a smaller (diluted) estimate of the strength of association between BPA and total testosterone concentrations: the true associations are likely to be much stronger.

The cross-sectional nature of the association reported here needs to be treated with caution. It is also theoretically possible that, for example, those with higher testosterone concentrations alter their diet in such a way as to increase BPA exposure, or as noted above that higher testosterone concentrations are themselves responsible for altering metabolism of BPA. It is unclear however why altered metabolism would alter our

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measure of 24 hour excretion systematically, as all BPA is thought to be excreted in the urine in humans sooner or later. We previously reported positive associations between urinary BPA and prevalence of cardiovascular disease (Lang et al. 2008, Melzer et al. 2010). The relationship between circulating testosterone and cardiovascular risk remains to be comprehensively established, although an increased risk of cardiovascular adverse events was recently reportedly in a trial of testosterone supplementation in older men (Basaria et al. 2010).

Future work needs to replicate the association found, and clarify the mechanisms involved. Showing that raised BPA levels precede the increase in testosterone concentrations would establish the temporal sequence of changes and exclude reverse causation. However, a concurrent change in testosterone levels with BPA exposure would remain biologically important. A large-scale exposure trial may be necessary to clarify the association identified, although the logistics and ethics of such a trial would require careful thought.

Conclusions

Mean daily exposure to BPA in an Italian adult population sample is in line with previous estimates from the USA, with wide variations around the mean. There is an association between higher daily excretion of BPA and total testosterone concentrations in men. The mechanisms involved in this possible endocrine disruption need clarification.

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Table 1. Characteristics of study sample: geometric mean and selected population percentiles of urinary Bisphenol A (BPA) concentrations and daily excretion. Grey lines denote BPA urinary excretion rate [UER, µg/day (95% C.I.)], white lines refer to urinary concentrations [ng/ml (95% C.I.)].

Variable	Number of observations	%	Geometric Mean [95% C.I.]	5 th perc.	10 th perc.	25 th perc.	50 th perc.	75 th perc.	90 th perc.	95 th perc.
All	715	-	5.63	2.1 (1.9-2.3)	2.6 (2.5-2.8)	3.7 (3.6-3.9)	5.6 (5.1-5.8)	8.3 (7.7-8.7)	11.8 (10.9-12.7)	16.4 (14.0-20.1)
	720	-	3.59	1.3 (1.2-1.4)	1.6 (1.5-1.7)	2.3 (2.1-2.4)	3.5 (3.3-3.7)	5.4 (5.0-5.9)	8.0 (7.4-9.5)	11.5 (10.3-13.7)
Gender										
Male	332	46.4	6.26	2.5 (2.0-2.7)	3.0 (2.6-3.3)	4.3 (3.9-4.6)	6.1 (5.7-6.8)	9.0 (8.3-9.7)	12.5 (11.7-15.4)	16.7 (14.5-23.7)
	334	46.4	4.02	1.5 (1.4-1.6)	1.8 (1.6-2.0)	2.4 (2.3-2.7)	3.9 (3.6-4.3)	6.3 (5.7-6.7)	9.8 (8.1-10.9)	13.0 (10.7-14.8)
Female	383	53.6	5.14	2.0 (1.8-2.2)	2.4 (2.2-2.6)	3.5 (3.2-3.7)	4.9 (4.5-5.3)	7.3 (6.7-8.2)	10.7 (9.9-12.3)	14.4 (12.2-20.4)
	386	53.6	3.25	1.1 (1.1-1.3)	1.4 (1.3-1.6)	2.1 (2.0-2.3)	3.2 (2.9-3.4)	4.7 (4.4-5.2)	7.2 (6.5-7.8)	11.0 (7.7-14.1)
Age-group										
20-40 yrs	109	15.2	6.61	2.6 (2.3-3.2)	3.2 (2.6-3.8)	4.7 (4.0-5.3)	6.7 (5.8-7.7)	8.9 (8.3-10.9)	12.5 (11.2-16.6)	16.9 (12.6-24.1)
	111	15.4	4.31	1.6 (1.2-2.1)	2.1 (1.6-2.3)	3.2 (2.4-3.6)	4.4 (4.0-4.8)	6.0 (5.6-6.8)	8.3 (7.0-12.0)	12.2 (8.4-17.4)
41-65 yrs	157	22.0	6.69	2.7 (2.2-3.2)	3.2 (2.8-3.6)	4.6 (4.0-5.0)	6.2 (5.6-6.9)	9.2 (8.1-10.3)	16.1 (11.3-21.3)	23.8 (16.7-40.7)
	157	21.8	3.95	1.4 (1.2-1.5)	1.5 (1.4-2.0)	2.4 (2.1-2.8)	3.7 (3.3-4.4)	5.8 (5.1-6.7)	9.6 (7.5-15.3)	16.7 (11.1-22.3)
66-74 yrs	449	62.8	5.10	1.9 (1.6-2.1)	2.4 (2.1-2.6)	3.5 (3.1-3.6)	4.9 (4.5-5.3)	7.4 (7.0-8.3)	10.9 (9.9-12.1)	14.2 (12.2-17.1)
	452	62.8	3.32	1.2 (1.1-1.3)	1.5 (1.3-1.6)	2.1 (2.0-2.3)	3.2 (2.9-3.4)	4.8 (4.4-5.6)	7.6 (7.0-8.9)	10.7 (9.3-12.8)

Table 2: Geometric means of BPA excretion ($\mu\text{g/day}$) by covariate status, plus age sex and study site adjusted regression estimates of association.

Variable	Obs.	%	Geometric Mean	[95% C.I.]	p-values for age, sex and site adjusted association
Years of education					
0 yrs	3	0.4	4.72	1.80 12.35	(dropped) *
1-5 yrs	364	50.9	5.06	4.75 5.39	
6-8 yrs	152	21.3	6.27	5.59 7.02	0.071
9-13 yrs	123	17.2	6.07	5.46 6.75	0.968
14-19 yrs	63	8.8	6.99	5.99 8.17	0.170
+20 yrs	10	1.4	5.84	3.82 8.94	0.576
Body Mass Index (BMI) categories					
Underweight (BMI 0-18.5)	4	0.6	2.74	1.29 5.81	(dropped) *
Normal (BMI 18.5-25)	215	30.1	5.67	5.22 6.16	
Overweight (BMI 25-30)	314	43.9	5.84	5.43 6.27	0.296
Obese I (BMI 30.1-34.9)	138	19.3	5.66	5.04 6.34	0.369
Obese II (BMI \geq 35)	32	4.5	4.85	3.94 5.98	0.738
Unknown	12	1.6	3.46	2.65 4.52	(dropped)
Smoking history					
Never	380	53.2	3.20	3.03 3.37	*
Former	171	23.9	3.76	3.50 4.05	0.259
Current	164	22.9	3.95	3.62 4.32	0.773
Continuous measures					
Waist circumference (cm)	715	-	$\beta = .0062$	(.0016 to .0108)	0.013
Weight (kg)	715	-	$\beta = .0064$	(.0023 to .0104)	0.003
Urinary creatinine concentration (mg/dl)	715	-	$\beta = -.0012$	(-.0025 to .0003)	0.116

Note: * is the base category against which the others are tested.

Table 3: Simple and fully adjusted regression models of the associations between BPA ($\mu\text{g/day}$) and 17 beta estradiol and testosterone concentrations for men

Hormone levels	n	Geometric Mean 95% CI	Age and study site adjusted		Fully adjusted	
			beta (95% CI), p-value	p-value	beta (95% CI), p-value	p-value
17-beta Estradiol (pg/ml)	293	12.89 (12.26 to 13.56)	-0.00004 (-0.0086 to 0.0085) p=0.992		0.0002 (-0.011 to 0.011) p=0.975	
Total Testosterone (ng/ml)	307	4.55 (4.42 to 4.69)	0.0237 (0.0006 to 0.0468) p= 0.044		0.046 (0.015 to 0.076) p=0.004	
Sex hormone binding globulin (nmol/ml)	316	80.84 (76.60 to 85.30)	-0.0009 (-0.0095 to 0.0076) p=0.830		0.0011 (-0.0075 to 0.0096) p=0.805	
Free testosterone (ng/dl)	316	4.72 (4.50 to 4.95)	.0081 (-0.0012 to 0.0175) p=0.089		.0088 (-0.0009 to 0.0185) p=0.075	

Note: full models adjusted for age, study site, smoking, BMI, weight, waist, and urinary creatinine concentration.

Table 4: Simple and fully adjusted regression models of the associations between BPA ($\mu\text{g/day}$) and 17 beta estradiol, testosterone and SHBG concentrations for women

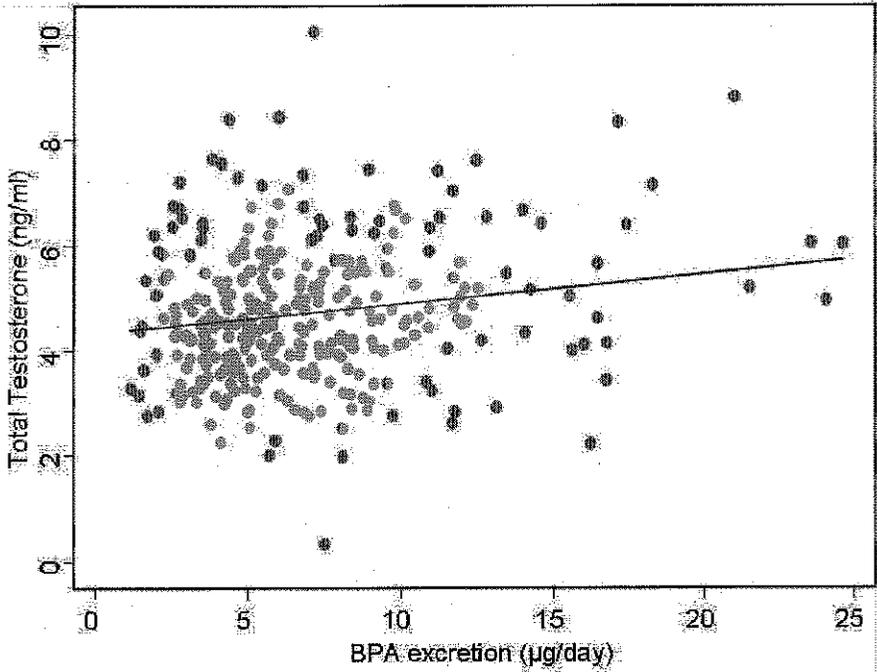
Hormone concentrations	n	Geometric Mean 95% CI	Age and study site adjusted		Fully adjusted	
			beta (95% CI), p-value	p-value	beta (95% CI), p-value	p-value
Pre-Menopause						
17-beta Estradiol (pg/ml)	57	22.4 (16.7 to 30.0)	-0.026 (-0.066 to 0.014) p=0.204		-0.022 (-0.066 to 0.0229) p=0.325	
Total Testosterone (ng/ml)	61	0.69 (0.61 to 0.77)	-0.004 (-0.015 to 0.007) p=0.451		-0.007 (-0.018 to 0.004) p=0.192	
Sex hormone binding globulin (nmol/ml)	60	134.3 (111.6 to 161.5)	0.029 (0.004 to 0.054) p=0.024		0.038 (0.013 to 0.063) p=0.004	
Post-Menopause						
17-beta Estradiol (pg/ml)	290	5.3 (4.8 to 5.7)	-0.002 (-0.010 to 0.005) p=0.516		-0.003 (-0.010 to 0.005) p=0.448	
Total Testosterone (ng/ml)	294	0.54 (0.49 to 0.59)	-0.0003 (-0.0036 to 0.0030) p=0.871		-0.001 (-0.004 to 0.002) p=0.555	
Sex hormone binding globulin (nmol/ml)	299	105.2 (98.8 to 112.1)	0.002 (-0.004 to 0.008) p=0.541		0.003 (-0.003 to 0.009) p=0.272	

Note: full models adjusted for age, study site, smoking, BMI, weight, waist, and urinary creatinine concentration.

Fig 1: Scatter plot of BPA excretion per day against total testosterone concentrations, with unadjusted linear regression line (BPA outlier values censored at <25 µg/day).

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138x108mm (150 x 150 DPI)