DRAFT
NTP BRIEF ON BISPHENOL A

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National Toxicology Program

National Institute of Environmental Health Sciences
National Institutes of Health
U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES

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Preface

The National Toxicology Program (NTP)[1] established the NTP Center for the Evaluation of Risks to Human Reproduction (CERHR) in June 1998. The purpose of the CERHR is to provide timely, unbiased, scientifically sound evaluations of the potential for adverse effects on reproduction or development resulting from human exposures to substances in the environment. The NTP-CERHR is headquartered at the National Institute of Environmental Health Sciences (NIEHS) and Dr. Michael Shelby is the director[2]

CERHR broadly solicits nominations of chemicals for evaluation from the public and private sectors. Chemicals are selected for evaluation based upon several factors including the following:

- potential for human exposure from use and occurrence in the environment
- extent of public concern
- production volume
- extent of database on reproductive and developmental toxicity studies

CERHR follows a formal process for review and evaluation of nominated chemicals that includes multiple opportunities for public comment. Briefly, CERHR convenes a scientific expert panel that meets in a public forum to review, discuss, and evaluate the scientific literature on the selected chemical. Public comment is invited prior to and during the meeting. The expert panel produces a report on the chemical's reproductive and developmental toxicities and provides its opinion of the degree to which exposure to the chemical is hazardous to humans. The panel also identifies areas of uncertainty and where additional data are needed. Expert panel reports are made public and comments are solicited.

Next, CERHR prepares the NTP Brief. The goal of the NTP Brief is to provide the public, as well as government health, regulatory, and research agencies, with the NTP's conclusions regarding the potential for the chemical to adversely affect human reproductive health or children's development. CERHR then prepares the NTP-CERHR Monograph, which includes the NTP Brief, the Expert Panel Report, and public comments on that report. The NTP-CERHR monograph is made publicly available on the CERHR web site and in hardcopy or CD from CERHR.

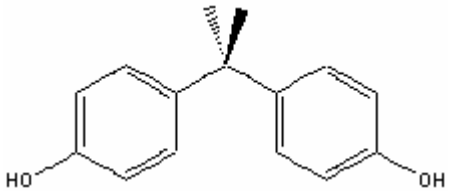
[1] NTP is an interagency program headquartered in Research Triangle Park, NC at the National Institute of Environmental Health Sciences, a component of the National Institutes of Health.

[2] Information about the CERHR is available on its web site (http://cerhr.niehs.nih.gov) or by contacting M.D. Shelby, Ph.D., Director, CERHR (P.O. Box 12233, MD EC-32, NIEHS, Research Triangle Park, NC 27709; telephone: 919-541-3455; facsimile: 919-316-4511; e-mail: shelby@niehs.nih.gov).

What is Bisphenol A?

Bisphenol A (BPA) is a chemical produced in large quantities for use primarily in the production of polycarbonate plastics and epoxy resins (Figure 1). It exists at room temperature as a white solid and has a mild "phenolic" or hospital odor. Polycarbonate plastics have many applications including use in certain food and drink packaging, e.g., water and infant bottles, compact discs, impact-resistant safety equipment, and medical devices. Polycarbonate plastics are typically clear and hard and marked with the recycle symbol "7" or may contain the letters "PC" near the recycle symbol. Polycarbonate plastic can also be blended with other materials to create molded parts for use in mobile phone housings, household items, and automobiles. Epoxy resins are used as lacquers to coat metal products such as food cans, bottle tops, and water supply pipes. Some polymers used in dental sealants or composites contain bisphenol A-derived materials. In 2004, the estimated production of bisphenol A in the United States was approximately 2.3 billion pounds, most of which was used in polycarbonate plastics and resins.

Figure 1. Chemical Structure of Bisphenol A ($C_{15}H_{16}O_2$; molecular weight 288.29)



CERHR selected bisphenol A for evaluation because it has received considerable attention in recent years due to widespread human exposures and concern for reproductive and developmental effects reported in laboratory animal studies. Bisphenol A is most commonly described as being "weakly" estrogenic; however, an emerging body of molecular and cellular studies indicate the potential for a number of additional biological activities. These range from interactions with cellular components that have unknown biological function to others that help mediate the actions of non-estrogenic hormones, such as androgens and thyroid hormones.

The NTP Brief on Bisphenol A is intended to be an environmental health resource for the public and regulatory and health agencies. It is not a quantitative risk assessment nor is it intended to supersede risk assessments conducted by regulatory agencies. The NTP Brief on Bisphenol A does not present a comprehensive review of the health-related literature or controversies related to this chemical. Only key issues and study findings considered most relevant for developing the NTP conclusions on concerns for potential reproductive and developmental human health effects of bisphenol A are discussed. Literature cited includes the most relevant studies reviewed in the CERHR Expert Panel Report on Bisphenol A and research articles published in the peer-reviewed literature subsequent to the deliberations of the expert panel.

Are People Exposed to Bisphenol A?[3]

[3] Answers to this and subsequent questions may be: *Yes, Probably, Possibly, Probably Not, No or Unknown*

Yes. The primary source of exposure to bisphenol A for most people is 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 [(*1*); reviewed in (*2, 3*)]. Bisphenol A can migrate into food from food and beverage containers with internal epoxy resin coatings and from consumer products made of polycarbonate plastic such as baby bottles, tableware, food containers, and water bottles. The degree to which bisphenol A migrates from polycarbonate containers into liquid appears to depend more on the temperature of the liquid than the age of the container, i.e., more migration with higher temperatures (*4*). Bisphenol A can also be found in breast milk (*5*). Short-term exposure can occur following application of certain dental sealants or composites made with bisphenol A-derived material such as bisphenol A-dimethyl acrylate (bis-DMA). Workers may be exposed during the manufacture of bisphenol A and bisphenol A-containing products.

Estimating human exposure to bisphenol A is generally done in one of two ways. Concentrations of bisphenol A can be measured directly in human blood, urine, breast milk, and other fluids or tissues ("biomonitoring"). Researchers can use biomonitoring information, such as the concentration of bisphenol A in urine, to estimate ("back calculate") a total intake that reflects all sources of exposure, both known and unknown. Scientists can also add, or aggregate, the amounts of bisphenol A detected in various sources, i.e., food and beverage, air, water, dust. The approach of aggregating exposure to estimate daily intake requires sources of exposure to be known and measured. In general, estimates based on biomonitoring are preferred for calculating total intake because all sources of exposure are integrated into the fluid or tissue measurement and do not have to be identified in advance. Estimates based on sources of exposure are useful to help discern the relative contributions of various exposure pathways to total intake.

The highest estimated daily intakes of bisphenol A in the general population occur in infants and children (Table 1). Infants and children have higher intakes of many widely detected environmental chemicals because they eat, drink, and breathe more than adults on a pound for pound basis. In addition, infants and children spend more time on the floor than adults and may engage in certain behaviors, such as dirt ingestion or mouthing of plastic items that can increase the potential for exposure.

Biomonitoring studies show that human exposure to bisphenol A is widespread (Table 2). The 2003-2004 National Health and Nutrition Examination Survey (NHANES III) conducted by the Centers for Disease Control and Prevention (CDC) found detectable levels of bisphenol A in 93% of 2517 urine samples from people 6 years and older (*6*). This study did not include children younger than 6 years of age. The CDC measured the "total" amount of bisphenol A in urine, a value that includes both bisphenol A and its metabolites. The CDC NHANES data are considered representative of exposures in the United States because of the large number of people included in the survey and the process used to select participants. In addition, the analytical techniques used by the CDC to measure bisphenol A are considered very accurate by the scientific community. Many smaller studies also report detection of bisphenol A in urine, blood, and other body fluids and tissues from people in the United States, Europe, and Asia [(*7-10*); studies published prior to mid-2007 are reviewed in (*2, 3, 11*)]. Concentrations of bisphenol A measured in breast milk and the blood of pregnant women in the United States are presented in Table 3.

It is helpful in interpreting the biomonitoring data for bisphenol A to understand how the body processes and excretes it once exposure occurs. Following ingestion, the majority of bisphenol A is quickly bound to glucuronic acid to produce bisphenol A-glucuronide, a metabolic process called glucuronidation that is carried out by enzymes primarily in the liver [reviewed in (*2*)]. Glucuronidation makes bisphenol A more soluble in water and, therefore, easier to eliminate in the urine and also minimizes its ability to interact with biological processes in the body. To a lesser extent, unconjugated parent (commonly referred to as "free") [4] bisphenol A is converted to other metabolites, primarily bisphenol A sulfate. Understanding the degree to which bisphenol A is metabolized is very important in determining whether bisphenol A poses a potential risk to human reproduction and development. While free bisphenol A and its major metabolites (bisphenol A-glucuronide and bisphenol A-sulfate) can all be measured in humans, only free bisphenol A is considered to be biologically active.

There is evidence in laboratory rodents that very young animals metabolize bisphenol A to its main biologically inactive metabolite, bisphenol A-glucuronide, less efficiently than adult animals (*12-14*). Neonatal rats do have some capacity to metabolize and eliminate bisphenol A; however, the enzyme systems that metabolize bisphenol A are not fully mature at this age and, as a result, neonatal rats have higher circulating concentrations of free bisphenol A in their blood compared to older animals given an equal exposure (*12*). There is also evidence for postnatal maturation of the corresponding enzymes in humans. Although a reduced ability or efficiency to glucuronidate is generally predicted for human fetuses and infants, this issue has not been specifically studied for bisphenol A [reviewed in (*2*)].

[4] Unmetabolized bisphenol A is commonly referred to as "free"; however, the majority of "free" bisphenol A circulating in human blood is bound to plasma proteins.

Table 1. Summary of Ranges of Estimated Daily Intakes in People Based on Sources of Exposure

Population	BPA µg/kg bw/day	Assumptions	References
Infant (0 – 6 months) Formula-fed	1 – 11*	• 1 assumes body weight of 4.5 kg and formula intake of 700 ml/day with 6.6 µg/L [maximum concentration detected in U.S. canned formula (*15, 16*)] (*2*) • 11 assumes body weight of 6.1 kg and formula intake of 1060 ml/day with (1) 50 µg/L bisphenol A/day migrating into formula from polycarbonate bottles (8.7 µg/kg bw/day); and (2) 14.3 µg bisphenol A/day ingested from powdered infant formula packed in food cans with epoxy linings (2.3 µg/kg bw/day) [0.143 kg powder/day (the amount of powder required to reconstitute a volume of formula of 1060 ml/day) containing 14.3 µg bisphenol A (100 µg bisphenol A/kg powder)]. 8.7 + 2.3 = 11 µg/kg bw/day (*17*)	(*2, 17-19*)
Infant (0 – 6 months) Breast-fed	0.2 - 1*	• 0.2 assumes body weight of 6.1 kg and breast milk intake of 1060 ml/day with 0.97 µg/L bisphenol A [maximum concentration of bisphenol A detected in Japanese breast milk samples (*20*)](*17*) • 1 assumes body weight of 4.5 kg and breast milk intake of 700 ml/day with 6.3 µg/L free bisphenol A [maximum concentration of free bisphenol A detected in U.S. breast milk samples (*5*)](*2*)	(*2, 17*)
Infant (6 – 12 months)	1.65 - 13*	• 1.65 assumes body weight of 8.8 kg with (1) 7 µg/L bisphenol A/day from formula intake of 700 ml/day with 10 µg/L (0.8 µg/kg bw/day); and (2) 7.6 µg/kg bisphenol A/day from ingestion of 0.38 kg canned food/day with 20 µg/kg (~0.85 µg/kg bw/day). 0.8 + 0.85 = 1.65 (*18*) • 13 assumes body weight of 7.8 kg, formula intake of 920 ml/day, and food consumption of 0.407 kg/day with (1) 50 µg/L bisphenol A migrating into formula from polycarbonate bottles (5.9 µg/kg bw/day); (2) 12.4 µg bisphenol A/day ingested from powdered infant formula packed in food cans with epoxy linings (1.6 µg/kg bw/day) [0.124 kg powder/day (the amount of powder required to reconstitute a volume of formula of 920ml/day) containing 12.4 µg bisphenol (100 µg bisphenol A/kg powder)]; (3) 40.7 µg bisphenol A/day ingested from canned food (5.2 µg/kg bw/day) [0.407 kg food/day containing 40.7 µg bisphenol A (100 µg bisphenol A/kg food)]; and (4) 2.04 µg bisphenol A/day migration from polycarbonate tableware (0.26, or ~ 0.3 µg/kg bw/day)[0.407 kg food/day containing 2.04 µg bisphenol A (5 µg bisphenol A/kg food)] 5.9 + 1.6 + 5.2 + 0.3 = 13.0 µg/kg bw/day (*17*)	(*16-19*)
Child (1.5 – 6 years)	0.043-14.7	• 0.043 is the mean (range: 0.018 – 0.071 µg/kg bw/day) based on individual body weight and measured concentrations of bisphenol in indoor and outdoor air, dust, soil, and liquid and solid food from daycare and home and the assumption of 100% absorption (*21*) • 14.7 assumes body weight of 14.5 kg and consumption of 2 kg canned food/day with (1) 200 µg bisphenol A/day ingested from canned food (~14 µg/kg bw/day) [2 kg food/day containing 200 µg bisphenol A (100 µg bisphenol A/kg food)]; and (2) 10 µg bisphenol A/day migration from polycarbonate tableware (~ 0.7 µg/kg bw/day)[2 kg food/day containing 10 µg bisphenol A (5 µg bisphenol A/kg food)] 14 + 0.7 = 14.7(*19*)	(*1, 17-19, 21, 22*)
Adult – general population	0.008 – 1.5**	• 0.008 assumes body weight of 74.8 kg and is based on measured concentrations of bisphenol A in 80 canned and bottled food items and a 24-hour dietary recall in ~4400 New Zealanders (*23*)	(*16-19, 22, 23*)

Population	BPA µg/kg bw/day	Assumptions	References
		• 1.5 assumes body weight of 60 kg and (1) 70 µg bisphenol A/day from canned food (1.2 µg/kg bw/day) [3 kg/day total consumption (1 kg solid food with 50 µg bisphenol A /kg and 2 L beverage with 10 µg bisphenol A /L)]; and 15 µg bisphenol A/day migration from polycarbonate tableware (0.25, or ~ 0.3 µg/kg bw/day)[3 kg food/day containing 15 µg bisphenol A (5 µg bisphenol A/kg food)] 1.2 + 0.3 = 1.5 µg/kg bw/day (*17*)	
Adult - occupational	0.043-100	• 0.043 is based on back calculating from a median urinary bisphenol A concentration of 1.06 µmol/mol creatinine (2.14 µg/g creatinine) from Hanaoka *et al.* (*24*). A daily intake of 0.043 µg/kg bw/day is based on the assumption of 1200 mg/day creatinine excretion (2.57 µg/day bisphenol excreted) and a body weight of 60 kg (*2*). • 100 is the maximal estimated exposures in U.S. powder paint workers based on time weighted averages of 0.001–1.063 mg/m^3, an inhalation factor of 0.29 m^3/kg day (*25*), 100% absorption from the respiratory system, and 8 hours worked per day (*2*).	(*2*, *19*, *25*)

*A study by Miyamoto *et al*. (*22*) reported much lower estimated intakes for infants (0.028 to 0.18 µg/kg bw/day); however, these estimates were excluded from the summary table because (1) insufficient detail was presented in the study to understand the assumptions used to derive these values, and (2) the authors assumed no bisphenol A in breast milk, an assumption not supported by data from the CDC (*5*) and Sun *et al.* (*20*).

** The European Union (*19*) calculated an extreme worst case scenario of ~ 9 µg/kg bw/day based on 1.4 µg/kg bw/day from food plus ~ 7 µg/kg bw/day from wine. The high estimated intake from wine (0.75 L wine/day with 650 µg bisphenol A /L = 325 µg bisphenol A/day, or ~7 µg/kg bw/day, from wine) was based on an extraction study conducted with an epoxy resin that is sometimes used to line wine vats. A study published subsequent to the evaluation by the European Union identified a maximum concentration of 2.1 µg bisphenol A /L in wine (*26*).

Table 2. Urinary Concentrations and Corresponding "Back Calculated" Daily Intakes of Bisphenol A in People (United States)

Population	Urinary Concentration of Total Bisphenol A (μg/L) median (25th – 95th percentile range)* (*6*)	Estimated Intake of Bisphenol A (μg/kg bw/day) median (25th – 95th percentile range)** (*27*)
All	2.7 (1.3 – 15.9)	0.0505 (0.0235 – 0.2742)
6-11 years	3.7 (1.7 – 16.0)	0.0674 (0.0310 – 0.3105)
12-19 years	4.2 (1.9 – 16.5)	0.0773 (0.0378 – 0.3476)
20-39 years	3.1 (1.5 – 15.4)	0.0563 (0.0272 – 0.2893)
40-59 years	2.4 (1.1 – 15.5)	0.0415 (0.0179 – 0.2335)
60+ years	1.9 (0.8 – 13.3)	0.0334 (0.0163 – 0.2331)
Female	2.4 (1.2 – 15.7)	0.0443 (0.0190 – 0.2705)
Male	3.2 (1.4 – 16.0)	0.0572 (0.0269 – 0.2778)

* The CDC data for ages 20-39 and 40-59 years were not presented in the study by Calafat *et al.* (*6*). Lakind *et al*. (*27*) obtained these values from data files available on the CDC website (http://www.cdc.gov/nchs/about/major/nhanes/nhanes2003-2004/lab03_04.htm). Lakind *et al*. (*27*) conducted a separate analysis of the CDC data and calculated mean and percentile values within 0.2 μg/L of those presented by Calafat *et al.* (*6*).

** Lakind *et al.* (*27*) assumed that daily intake of bisphenol A was equivalent to daily excretion. Daily excretion was calculated by multiplying the urine concentration of bisphenol A (μg/L) by 24-hour urinary output volume. Daily urinary volume was assumed to be 600 ml for children aged 6-11 years, 1200 for adult females, and 1600 for adult males. Body weight data from the 2003-2004 NHANES database was used to calculate daily intake adjusted for body weight.

Table 3. Blood and Breast Milk Biomonitoring of Bisphenol A in People (United States)

Biological Medium	Population (sample size)	Free BPA (μg/L) mean or median [range]	Total BPA (μg/L) mean or median [range]	Reference
Blood	Pregnant women (40)	mean: 5.9 [0.5 - 22.4]		(*10*)
Breast milk	Lactating women (20)	mean: 1.3; median: 0.4 [< 0.3 (LOD) - 6.3]	mean: 1.3; median: 1.1 [< 0.3 (LOD) - 7.3]	(*5*)

LOD = limit of detection

Can Bisphenol A Affect Human Development or Reproduction?

Possibly. Although there is no direct evidence that exposure of people to bisphenol A adversely affects reproduction or development, studies with laboratory rodents show that exposure to high dose levels of bisphenol A during pregnancy and/or lactation can reduce survival, birth weight, and growth of offspring early in life, and delay the onset of puberty in males and females. These effects were seen at the same dose levels that also produced some weight loss in pregnant animals ("dams"). The administered dose levels associated with delayed puberty (≥ 50 mg/kg bw/day), growth reductions (≥ 300 mg/kg bw/day), or survival (≥ 500 mg/kg bw/day) are far in excess of the highest estimated daily intakes of bisphenol A in children (< 0.0147 mg/kg bw/day), adults (< 0.0015 mg/kg bw/day), or workers (0.100 mg/kg bw/day) (Table 1). These "high" dose effects of bisphenol A are not considered scientifically controversial and provide *clear evidence* of adverse effects on development in laboratory animals.

In addition to effects on survival and growth seen at high dose levels of bisphenol A, a variety of effects related to neural and behavior alterations, precancerous lesions in the prostate and mammary glands, altered prostate gland and urinary tract development, and early onset of puberty in females have been reported in laboratory rodents exposed during development to much lower doses of bisphenol A (≥ 0.0024 mg/kg bw/day) that are more similar to human exposures. In contrast to the "high" dose developmental effects of bisphenol A, there is scientific controversy over the interpretation of the "low" dose findings. When considered together, the results of "low" dose studies of bisphenol A provide *limited evidence* for adverse effects on development in laboratory animals (see **Figures 2a & 2b**).

Recognizing the lack of data on the effects of bisphenol A in humans and despite the limitations in the evidence for "low" dose effects in laboratory animals discussed in more detail below, the possibility that bisphenol A may alter human development cannot be dismissed (see **Figure 3**).

Supporting Evidence

The NTP finds that there is clear evidence of adverse developmental effects at "high" doses of bisphenol A in the form of fetal death, decreased litter size, or decreased number of live pups per litter in rats (≥ 500 mg/kg bw/day) (*28, 29*) and mice (≥ 875 mg/kg bw/day) (*30-32*), reduced growth in rats (≥ 300 mg/kg bw/day) (*28, 29*) and mice (≥ 600 mg/kg bw/day) (*30, 31, 33*), and delayed puberty in male mice (600 mg/kg bw/day) (*33*), male rats (≥ 50 mg/kg bw/day) (*29, 34*) and female rats (≥50 mg/kg bw/day) (*29, 35*).

In addition to these "high" dose effects on survival and growth, the NTP recognizes that there are studies that provide evidence for a variety of effects at much lower dose levels of bisphenol A related to neural and behavioral alterations in rats and mice (≥ 0.010 mg/kg bw/day) (*36-42*), preneoplastic lesions in the prostate and mammary gland in rats (0.010 mg/kg bw/day and 0.0025 mg/kg bw/day, respectively) (*43-45*), altered prostate and urinary tract development in mice (0.010 mg/kg bw/day) (*46*), and early onset of puberty in female mice (0.0024 and 0.200 mg/kg bw/day) (*40, 47*).

These "low" dose findings in laboratory animals have proven to be controversial for a variety of reasons including concern for insufficient replication by independent investigators, questions on the suitability of various experimental approaches, relevance of the specific animal model used for evaluating potential human risks, and incomplete understanding or agreement on the potential adverse nature of reported effects. These issues have been extensively addressed elsewhere (*2, 48-52*) and were considered by the NTP when evaluating the bisphenol A literature.

How Was This Conclusion Reached?

Scientific decisions concerning health risks are generally based on what is known as the "weight-of-evidence." In the case of bisphenol A, evidence from the limited number of studies in humans exposed to bisphenol A is not sufficient to reach conclusions regarding possible developmental or reproductive hazard. In contrast, there is a large literature of laboratory animal studies. These include studies of traditional designs carried out to assess the toxicity of bisphenol A, as well as a wide variety of studies examining the possibility that exposure to "low" doses of bisphenol A, defined in the NTP Brief on Bisphenol A as ≤ 5 mg/kg bw/day (*53*), during critical periods of development might result in adverse health outcomes later in life due to its estrogenic or other biological properties. Many of these latter studies were designed not as toxicology studies but rather to probe very specific experimental questions and their results are not always easily interpreted with regard to how they contribute to the weight-of-evidence for human health risks.

Many of the laboratory animal studies of bisphenol A have technical or design shortcomings or their reports do not provide sufficient experimental details to permit an assessment of technical adequacy (*2*). As discussed in more detail below, the NTP did not establish strict criteria for determining which studies from the bisphenol A literature to consider for the evaluation. Rather, in an effort to glean information that might contribute to understanding the numerous reported effects of bisphenol A, NTP evaluated many individual study reports. Attention was paid to issues of sample size, control for litter effects, and various other aspects of experimental design; however, experimental findings were initially evaluated in relation to their biological plausibility and consistency across studies by multiple investigators. Studies were then evaluated as to their adequacy of experimental design and the likelihood that any inconsistent outcomes resulted from differences or shortcomings in experimental design. The NTP considered several overarching issues when evaluating the bisphenol A literature:

- Are the *in vivo* effects reproducible and/or biologically plausible?

Two issues become evident when considering the topic of reproducibility of effects in the bisphenol A literature. In some cases, the reproducibility of certain effects has been questioned because attempts at replication by other researchers using similar experimental designs did not necessarily produce consistent findings. This leads to reduced confidence in the utility of the effect for identifying a hazard. Numerous reasons have been suggested to explain the inconsistent findings including differences in sensitivity of the rodent model, i.e., species, strain, breeding stock, the author's funding source, the degree of laboratory expertise, and variations in diet,[5] animal husbandry, and route of administration. However, it is not known if these factors

[5] Understanding the impact of variations in dietary phytoestrogen content in laboratory animal studies of estrogenic compounds, including bisphenol A, is an active area of inquiry (*54*). Recent research suggests that bisphenol A may

account for the inconsistencies. In other cases, particularly for findings based on studies with very specific experimental questions, variations in experimental design are large enough to conclude that the reproducibility of the finding is essentially unknown. A number of these effects have not been addressed in traditional toxicity studies carried out to assess the toxicity of bisphenol A. Typically the safety studies do not probe for potential organ effects with the same degree of specificity or detail as those studies with specific experimental questions. The NTP evaluated the biological plausibility of findings with unknown reproducibility in light of supporting data at the mechanistic, cellular, or tissue level.

Another issue is that the "low" dose studies generally have not tested higher dose levels of bisphenol A, i.e., > 1 mg/kg. Testing over a wide range of dose levels is necessary to adequately characterize the dose-response relationship. Typically, effects are easier to interpret when the dose-response curve is monotonic and the incidence, severity, or magnitude of response increases as the dose level increases. Effects that have biphasic, or non-monotonic dose response curves, are well documented in toxicology, endocrinology and other scientific disciplines (*56, 57*), but can be more difficult to interpret, which often limits their impact in risk assessments or other health evaluations. Testing higher dose levels may also identify additional effects that aid in interpreting the "low" dose finding with respect to potential health risk.

- Do the *in vivo* effects represent adverse health findings in laboratory animals and/or humans?

A general limitation in the "low" dose literature for bisphenol A is that many studies have addressed very specific experimental questions and not necessarily established a clear linkage between the "low" dose finding and a subsequent adverse health impact. For example, when an effect is observed in fetal, neonatal, or pubertal animals, investigations may not have been conducted to determine if the effect persists or manifest as a clear health effect later in life. Establishing a linkage to an adverse health impact is important because many of the "low" dose findings can be described as subtle, which can make them difficult to utilize for risk assessment purposes. An additional factor in considering the adversity of a finding is determining if the experimental model is adequate for predicting potential human health outcomes.

- How should studies that use a non-oral route of administration be interpreted?

Because the majority of exposure to bisphenol A occurs through the diet (*1*), laboratory animal studies that use the oral route of administration are considered the most useful to assess potential effects in humans. However, a large number of the laboratory animal studies of bisphenol A have used a subcutaneous route of administration to deliver the chemical, either by injection or mini-pumps that are implanted under the skin. The consideration of these studies in health evaluations of bisphenol A has proven controversial (*2, 58*). There is scientific consensus that doses of bisphenol A administered orally and subcutaneously cannot be directly compared in adult laboratory animals because the rate of metabolism of bisphenol A differs following oral and non-oral administration. There is also consensus that fetal and neonatal rats do not metabolize bisphenol A as efficiently as adult rats at a given dose because the enzyme systems that are responsible for the metabolism of bisphenol A are not fully mature during fetal or neonatal life.

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alter DNA methylation (an epigenetic mechanism to alter phenotype) following exposure during development and that this effect may be offset by dietary exposure to methyl donors or the phytoestrogen genistein (*55*).

However, there is scientific debate on whether the reduced metabolic capability of neonatal rats is sufficient to adequately metabolize low doses of bisphenol A.

In adult rats and monkeys, bisphenol A is metabolized to its biologically inactive form, or glucuronidated, more quickly when administered orally than by a non-oral route, e.g., subcutaneously, intraperitoneally, or intravenously (*59-61*). This is because bisphenol A administered orally first passes from the intestine to the liver where it undergoes extensive conjugation primarily with glucuronic acid before reaching the systemic circulation ("first pass metabolism"). Because non-oral administration bypasses the liver, and therefore first pass metabolism, these routes of dosing in adult rats and monkeys result in higher circulating concentrations of biologically active, free bisphenol A compared to oral administration. Although not tested directly in adult laboratory mice, the impact of first pass metabolism is predicted to be similar. Thus, a subcutaneous dose is expected to have a greater biological effect than the same dose delivered by mouth in adult laboratory animals, including in the offspring of dams treated with bisphenol A during pregnancy.

Studies that administer bisphenol A through non-oral routes are most useful for human health evaluations when information on the fate, e.g., half-life, and concentration of free bisphenol A in the blood or other tissue is also available. For example, if the peak and average daily concentrations of free bisphenol A in blood were measured following non-oral administration, these values could then be compared to levels of free bisphenol measured in rodent studies where bisphenol A is administered orally or to levels measured in humans. However, none of the reproductive and developmental toxicity studies that treated animals by non-oral routes of administration determined the circulating levels of free bisphenol A or its metabolites. As a result, studies that treat laboratory animals using non-oral routes of administration have often been considered of no or of limited relevance for estimating potential risk to humans (*2, 19, 48*).

As discussed previously (see "Are People Exposed to Bisphenol A?"), fetal and neonatal rats do not metabolize bisphenol A as efficiently as the adult and, as a result, have higher circulating concentrations of free bisphenol A for some period of time compared to adults receiving the same dose (*12-14*). The peak concentrations of free bisphenol A in the blood of 4-day old male and female rat pups orally dosed with 10 mg/kg are 2013 and 162-times higher than the peak blood levels measured in male and female adult rats treated with the same mg/kg dose (*12*). A measure of how long it takes the body to eliminate free bisphenol A, referred to as "half-life," was also slower at this dose in neonatal rats: > 6.7 hours in male or female pups compared to well under an 1 hour in adult animals (*12*). Thus, for a given administered dose, blood levels of bisphenol A are higher in neonatal rats than in adults, and remain so longer following exposure. However, neonatal rats do have the ability to metabolize bisphenol A as indicated by the presence of bisphenol A glucuronide in the blood and the inability to detect the free form within the measurement sensitivity of the assay by 12 to 24-hours after treatment in females and males respectively (*12*).

Neonatal rats appear to be able to more efficiently metabolize bisphenol A when given at lower dose levels than at higher dose levels. Although Domoradzki *et al.* (*12*) also treated neonatal and adult animals with a lower dose level of bisphenol A, 1 mg/kg, making a direct comparisons based on age at exposure was not possible at that dose because free bisphenol A was too low to

be quantified in the blood of adults. However, in 4-day old male and female rats treated with 1 mg/kg of bisphenol A, 98 – 100% of administered bisphenol A was detected as bisphenol A-glucuronide[6] compared to 71 – 82% at 10 mg/kg, i.e., a smaller proportion of administered bisphenol A is glucuronidated at 10 mg/kg compared to 1 mg/kg. This would be expected when the limited capacity of young animals to metabolize bisphenol A is overwhelmed by dose levels of the compound. These data suggest more efficient metabolism by neonatal rats at 1 mg/kg compared to 10 mg/kg and imply that the age at exposure differences described above may be less profound in the "low" dose range (≤ 5 mg/kg bw/day).

Taken together these data indicate that, compared to adults at a given dose, neonatal rats (and presumably mice) metabolize bisphenol A more slowly and suggest that differences in circulating levels of free bisphenol A arising from oral and subcutaneous routes of administration as a result of "first-pass metabolism" are reduced in fetal or infant animals compared to adults. This prediction is supported by a recent study that did not detect differences in the blood concentration of free bisphenol A as a function of route of administration (oral versus subcutaneous injection) in 3-day old female mice following treatment with either 0.035 or 0.395 mg/kg of bisphenol A (*58*).

While more research in this area is warranted, data from studies where bisphenol A was given by subcutaneous injection were considered as useful in the NTP evaluation as oral administration when treatment occurred during infancy when the capacity to metabolize bisphenol A is low. Studies in adult animals, including pregnant dams, that administered bisphenol A by subcutaneous injection or by a subcutaneous mini-pump were considered informative for identifying biological effects of bisphenol A but not for quantitatively comparing exposures in laboratory animals and humans.

- What is the impact of limitations in experimental design and how should studies with these limitations be interpreted?

The impact on study interpretation due to limitations in experimental design has been a significant point of discussion for bisphenol A, especially for the issues of (1) small sample size, (2) a lack of experimental or statistical control for litter effects, and (3) failure to use a positive control (*2*, *62*).

In general, studies with larger sample sizes will have more power to detect an effect due to bisphenol A exposure than studies with small sample sizes. For this reason, "negative" results from small sample size studies are viewed with caution. On the other hand, "negative" results from studies with larger sample sizes are usually considered more credible (*63*). However, there is no single sample size that can be identified as appropriate for all endpoints. The ability to detect an effect is affected by the background incidence, e.g., tumor or malformation rates in control animals, variability of a particular endpoint, and the magnitude of the effect. A sample size of at least six may be reasonable for many endpoints with low or moderate degrees of variability, such as body weight, but could be insufficient to detect statistically significant differences in endpoints with a higher degree of variability such as hormone level or sperm

[6] Based on percentage of plasma area under the curve (AUC) for radioactivity that was bisphenol A glucuronide.

count, or that occur infrequently such as malformations or tumor formation. These factors can make consistent detection of relatively small changes especially difficult on endpoints that have a high degree of inherent variability.

Lack of statistical or experimental control for litter effects was perhaps the single most common technical shortcoming noted in the developmental toxicity studies evaluated by the CERHR Expert Panel for Bisphenol A (*2*). Adequate control for litter effects when littermates are used in an experiment is considered essential in developmental toxicology. In 2000, the NTP co-sponsored a workshop with the U.S. Environmental Protection Agency referred to as the "Low Dose Endocrine Disruptors Peer Review." As part of the peer review, a group of statisticians reanalyzed a number of "low" dose studies (*63*). Based on studies that used littermates, they determined that litter or dam effects were generally present such that pups within a litter were found to respond more similarly than pups from different litters. The overall conclusion on this issue was that "[f]ailure to adjust for litter effects (e.g., to regard littermates as independent observations and thus the individual pup as the experimental unit) can greatly exaggerate the statistical significance of experimental findings." Studies that did not adequately control for litter effects were given less weight in the NTP evaluation and were generally only used as supportive material.

The NTP concurs with the opinion of several scientific panels that positive control groups can be very useful to evaluate the sensitivity and performance of a given experimental model (*2, 52, 63*). However, the NTP does not consider use of a positive control to be a required study design component particularly in animal model systems that are well characterized regarding the background incidence of "effects" and their variability. For bisphenol A studies, potent estrogens, such as diethylstilbestrol, ethinyl estradiol, 17β-estradiol, and estradiol benzoate, are the most commonly used positive control chemicals given bisphenol A's historical classification as a weak estrogen. Failure to obtain predicted responses with these chemicals is generally interpreted as a "failed" experiment, perhaps reflecting the selection of a relatively insensitive animal or experimental model or insufficient chemical challenge. Studies where no responses are observed in the positive control group have generally contributed less weight to evaluations of bisphenol A (*2, 52*). The significance of a "failed" positive control for bisphenol A varies from endpoint to endpoint and reflected more negatively on a study in the NTP evaluation when the predicted effect on reproductive tissue or function was not observed at dose levels that should be sufficiently high to produce an effect.

Although potent estrogens are used as positive controls for bisphenol A, an increasing number of molecular or cell-based ("*in vitro*") studies suggest that interpreting the toxicological effects of bisphenol A solely within the context of their consistency with a classic estrogenic mechanism of action, or even as a selective estrogen receptor modulator (SERM),[7] is overly simplistic. In addition to binding to the nuclear estrogen receptors ERα and ERβ, bisphenol A interacts with a variety of other cellular targets [reviewed in (*2, 64*)] including binding to a non-classical membrane-bound form of the estrogen receptor (ncmER) (*65-67*), a recently identified orphan nuclear receptor called estrogen-related receptor gamma ERR-γ (*68-72*), a seven-transmembrane estrogen receptor called GPR30 (*73*), and the aryl hydrocarbon receptor (AhR) (*74, 75*).

[7] A selective estrogen receptor modulator, or SERM, is a compound that binds nuclear estrogen receptors and acts as an estrogen agonist in some tissues and as an estrogen antagonist in other tissues.

Several *in vitro* studies show that bisphenol A can act as an androgen receptor antagonist (*74, 76-82*) and is reportedly mitogenic in a human prostate carcinoma cell line through interactions with a mutant tumor-derived form of the androgen receptor (*83*). Bisphenol A also interacts with thyroid hormone receptors (TRs) and, based on *in vitro* studies, is reported to either inhibit TR-mediated transcription (*84*), inhibit the actions of triiodothyronine (T3) or its binding to TRs (*85, 86*); or stimulate cell proliferation in a thyroid hormone responsive cell line (*87*). One *in vivo* study suggests that bisphenol A acts as a selective TRβ antagonist (*88*). Bisphenol A may also inhibit activity of aromatase, the enzyme that converts testosterone to estradiol (*74, 89*).

The toxicological consequences of the non-nuclear estrogen receptor interactions identified so far are unclear. In some instances, the physiologic role of the receptor is unknown or not well characterized, i.e., ERR-γ, GPR30, which makes interpreting the consistency of the data impossible with respect to the implicated mechanism based on the cellular or molecular studies and the observed *in vivo* toxicology. However, even when the physiological effects are generally understood, e.g., AhR or AR binding, aromatase function, scientists can only speculate as to the possible *in vivo* impacts when multiple receptor or other cellular interactions are considered together. Nevertheless, the identification of a growing number of cellular targets for bisphenol A may help explain toxicological effects that are not considered estrogenic or predicted simply based on the lower potency of bisphenol A compared to estradiol. Effects mediated through the ncmER are of interest because of its role in regulating pancreatic hormone release and because bisphenol A has been shown to activate this receptor *in vitro* at a concentration of 1 nM, which is similar to the active concentration of the potent estrogen diethylstilbestrol (*65, 67*).

Human Studies

Only a very small number of studies have looked at associations between bisphenol A exposure and disorders of reproduction or developmental effects in humans [(*10, 90, 91*), studies prior to mid-2007 reviewed in (*2, 3*)]. The human studies have looked at the relationship between urine or blood concentrations of total or free bisphenol A and a variety of health measures including levels of certain hormones that help regulate reproduction (*24, 92*), markers of DNA damage (*93*), miscarriage (*94*), chromosomal defects in fetuses (*95*), fertility and obesity in women (*90, 96, 97*), effects on the tissue that lines the uterus ("endometrium") (*90, 98*), polycystic ovary syndrome (*92, 97*), and birth outcomes and length of gestation (*10, 91*).

In these studies, there are reports of associations between higher urine or blood concentrations of bisphenol A and lower levels of follicle-stimulating hormone in occupationally exposed men (*24*), higher levels of testosterone in men and women (*92, 97*), polycystic ovary syndrome (*92, 97*), recurrent miscarriage (*94*), and chromosomal defects in fetuses (*95*). In addition, one study reported that patients with endometrial cancer and complex endometrial hyperplasia had lower blood levels of bisphenol A than healthy women and women with simple endometrial hyperplasia (*98*). Bisphenol A was not associated with decreased birth weight or several other measures of birth outcome in two recent studies (*10, 91*). Drawing firm conclusions about potential reproductive or developmental effects of bisphenol A in humans from these studies is difficult because of factors such as small sample size, cross-sectional design, lack of large variations in exposure, or lack of adjustment for potential confounders. However, the NTP

Expert Panel on Bisphenol A (*2*) concluded that several studies collectively suggest hormonal effects of bisphenol A exposure (*24, 92, 97*) including one in occupationally exposed male workers likely exposed through multiple routes including inhalation (*24*).

The NTP concurs with findings of the recent evaluations (*2, 3*) that while these studies may suggest directions for future research, there is currently insufficient evidence to determine if bisphenol A causes or does not cause reproductive toxicity in exposed adults. There is also insufficient evidence in humans to determine if bisphenol A does or does not cause developmental toxicity when exposure occurs prenatally or during infancy and childhood.

Laboratory Animal Studies

In contrast to the limited literature evaluating possible effects of bisphenol A in humans, the scientific literature on the toxic effects of bisphenol A in laboratory animals is extensive and expanding. For example, between February 2007, the cut-off date for literature included in the CERHR Bisphenol A Expert Panel Report, and April 11, 2008, more than 400 new articles related to bisphenol A were identified by PubMed search. All new studies related to the potential reproductive and developmental effects of bisphenol A were considered during preparation of the draft NTP Brief on Bisphenol A. However, only those studies that were considered the most informative for developing NTP conclusions are cited in the Brief. In addition to the new literature cited, many key studies reviewed in the expert panel report are cited herein.

Reproductive Toxicity Studies

The reproductive toxicity studies of bisphenol A include assessment of fertility, sperm counts, estrous cycling, and growth or cellular damage in reproductive tissues. Reproductive toxicity can be studied in animals exposed during adulthood, during development, or both. Conclusions on reproductive toxicity presented in this section of the NTP Brief on Bisphenol A are limited to the assessment of fertility in laboratory animals, regardless of when exposure occurred, and other indicators of reproductive effects in animals exposed only during adulthood. Assessments of aspects of the reproductive system other than fertility in animals exposed during development are discussed under the headings of "High" Dose and "Low" Dose Developmental Toxicity Studies below.

Studies show that bisphenol A does not reduce fertility in laboratory animals exposed in adulthood and/or during developmental at dose levels up to 500 mg/kg bw/day in rats (*29, 99*). Fertility may be negatively impacted at higher dietary doses (≥ 875 mg/kg bw/day) in mice exposed as adults as indicated by a decreased number of litters per breeding pair (*32*), although two multigenerational reproductive toxicity studies did not report effects on fertility in mice at doses up to 1669 – 1988 mg/kg bw/day (*31, 33*). There are occasional reports of decreased fertility in smaller sample size studies of rodents exposed to much lower dose levels of bisphenol A during adulthood, such as oral treatment with 0.025 and 0.100 mg/kg bw/day in male mice (*100*). In the Al-Hiyasat *et al*. study, decreased pregnancy rates and increased incidence of resorptions in untreated female mice were attributed to effects in treated adult males, i.e., reductions in the number of testicular or epididymal sperm and hypothesized impaired sperm quality. However, the magnitude of the impact on weight-corrected testicular or epididymal

sperm number, ~16 to 37%, is not generally considered severe enough to account for the observed pregnancy rate decrease of ~33 to 40%.[8]

At high oral dose levels, adult exposure to bisphenol A caused reproductive toxicity in the form of altered estrous cycling in female rats (≥ 600 mg/kg bw/day)[9] (*102*) and cellular effects on the testis of male rats (235 mg/kg bw/day) (*103*). In addition, more subtle effects on maternal behavior, i.e., decreased duration of licking and grooming of pups, are reported at a lower oral dose in treated adult female rats (0.04 mg/kg bw/day) (*104*).

"High" Dose Developmental Toxicity Studies (> 5 mg/kg bw/day)

Results from developmental toxicity studies in mice and rats show adverse effects on pup survival and growth following maternal exposure to dose levels of bisphenol A defined by the NTP as "high" (> 5 mg/kg bw/day). In rats, a ~ 20 - 36% decrease in the number of pups per litter is reported following maternal dosing with ≥ 500 mg/kg bw/day (*28*, *29*). Increases in fetal death and post-implantation loss are seen in rats treated with 1000 mg/kg bw/day during pregnancy (*28*). Reductions in fetal weight or growth during postnatal life occur at oral dose levels of ≥ 300 mg/kg bw/day in rats (*28*, *29*). In mice, developmental toxicity is generally reported at higher oral doses in the form of fetal death, decreased number of live pups, reduced fetal or pup body weight at ≥875 mg/kg bw/day (*30*-*32*), and reductions in body weight during postnatal life in the F1 generation (but not the F2 generation) at 600 mg/kg bw/day (*33*). Fetal death in mice has also been observed in a recent study that reported embryo lethality following subcutaneous dosing with 10 mg/kg bw/day bisphenol A to pregnant mice (*105*). Occasionally, decreases in pup survival have been reported at much lower oral dose levels, such as 0.0024 mg/kg bw/day in mice (*106*). However, this effect is not typically reported at oral doses in this range even in studies from the same laboratory using a similar dosing regimen and the same source of mice (*107*).

Delayed onset of puberty (assessed by day of vaginal opening) has been reported in the female offspring of rats orally treated with bisphenol A at 50 mg/kg bw/day during gestation (*35*) or 500 mg/kg bw/day during gestation and lactation (*29*). In the study by Tyl *et al.* (*29*), this effect has been attributed to a decrease in body weight also observed at that dose and has not necessarily been considered a direct developmental effect (*19*). However, decreased body weight was not observed in females at the dose where delayed vaginal opening was reported by Tinwell *et al.* (*35*). This high dose effect of delayed vaginal opening is not the predicted effect of exposure to an estrogenic compound. It is worth noting that Tinwell *et al.* (*35*) did not detect any difference in onset of puberty in female rats when age at first estrous assessed by vaginal smear was used as the marker of puberty. Other "high" dose studies report no effect on onset of puberty in female rats exposed during gestation and lactation at maternal oral doses ranging from 3.2 to ~1000 mg/kg bw/day (*108*-*110*). One "high" dose study reported an accelerated onset of puberty in female rats following subcutaneous injection of bisphenol A during early post-natal life at 105 and 427 mg/kg bw/day (*111*). Delayed puberty in male rats treated during development has also

[8] Sperm counts in laboratory rodents and rabbits generally have to be severely impacted to cause infertility. Rats may still be fertile with a 90% reduction in sperm count (*101*).

[9] Animals were treated with 1000 mg/kg bw/day for 1-week and then the dose was reduced to 600 mg/kg for 22-25 additional days.

been reported at oral doses of ≥ 50 mg/kg bw/day (*29, 34*). This effect was associated with decreased body weight in the study by Tyl *et al.* (*29*), but not in the study by Tan *et al.* (*34*). A delay in puberty of 1.8 days has also been reported in male mice at 600 mg/kg bw/day in a 2-generation reproductive toxicity study (*33*).

With the exception of a possible morphological alteration of the urethra (discussed below) (*46*), bisphenol A has not been shown to cause malformations, such as skeletal birth defects or abnormally shaped or absent organs, in rats or mice at oral doses up to 1000 and 1250 mg/kg bw/day, respectively (*28, 30*). An indication of a possible developmental delay, apparent delayed bone formation ("ossification"), was reported at an oral dose level of 1000 mg/kg bw/day (*28*). A more subtle effect, cellular changes in the liver, in developmentally exposed animals has been reported at ≥ 50 mg/kg bw/day (*33*).

<u>"Low" Dose Developmental Effects in Laboratory Animals (≤ 5 mg/kg bw/day)</u>

- Neural and Behavioral Alterations

The NTP concurs with the CERHR Expert Panel on Bisphenol A that there is a sufficiently consistent body of literature to suggest that perinatal or pubertal exposure to "low" doses of bisphenol A causes neural and behavioral alterations in rats and mice, especially related to the development of normal sex-based differences between males and females ("sexual dimorphisms" or "sexually dimorphic").

Research on the effects of bisphenol A on the brain and behavior does not have as long a history as the assessment of reproductive tissues, but is now an active area of study that has been growing quickly in the past few years. Currently, the literature is composed of a collection of findings based on behavioral assessments, morphometric and cell-based measurements of the brain of laboratory animals, and *in vitro* studies to identify molecular and cellular targets and mechanisms of action. From these studies themes are emerging that suggest exposures to bisphenol A can produce a loss or reduction of sexual dimorphisms in non-reproductive behaviors and in certain regions of the brain as well as effects on the dopaminergic system. Neural effects are also implicated from mechanistic studies that show bisphenol A can interfere with thyroid hormone signaling.

Sexual dimorphisms include differences in the size, cellular composition, or molecular expression patterns of specific regions or structures in the brain. The studies detecting bisphenol A-induced changes in sexually dimorphic brain structures generally report a reduction or loss of sexual dimorphisms, for example, in the locus ceruleus (a brain region involved in mediating responses to stress) (*112, 113*), and the bed nucleus of the stria terminalis (involved in regulating emotional behavior) (*114*). Similar effects are reported in some, but not all, studies (*115-117*) of the anteroventral periventricular nucleus, a brain region that provides input to gonadotropin-releasing hormone neurons involved in regulating ovulation. The lowest administered doses delivered to either pregnant dams or neonatal animals associated with these effects range from ~0.03 mg/kg bw/day (oral) (*113*), 0.000025 mg/kg bw/day (subcutaneous mini-pump) (*116*) to ~100 mg/kg bw/day (subcutaneous injection) (*115*). Changes are not reported for all sexually dimorphic structures. One well-known sexually dimorphic structure reportedly not affected even

at doses up to 320 mg/kg bw/day in rats is the sexually dimorphic nucleus in the preoptic area (SDN-POA), a brain region that has a homologue in humans and is known to be modified by gonadal hormones during perinatal life (*108, 110, 112, 113, 117, 118*). Interpreting the potential human health or behavioral significance of effects on sexually-dimorphic brain regions can be difficult. For example, the bed nucleus of the stria terminalis is described as being responsive to reproductive hormones and generally involved in regulating emotional behavior (*119*), but the specific functions of this brain region in rats, and therefore the impact of loss of sexual dimorphism, remain unclear.

Effects on behavior have been assessed by a wide variety of experimental tests. Reported behavioral changes in rats or mice relate to play (*120*), maternal behavior (*36, 104*), aggression (*121, 122*), cognitive function (*123*), motor activity (*124, 125*), exploration (*38*), novelty-seeking (*38*) (*37, 126*), impulsivity (*126*), reward response (*37, 126-128*), pain response (*129*), anxiety and fear (*38, 40, 42, 130*), and social interactions (*131*). Many of these behaviors, including activity, anxiety, exploration, novelty seeking are sexually dimorphic to some degree. The lowest oral dose associated with behavioral changes is 0.01 mg/kg bw/day (via treatment to the pregnant dam) (*36-38*) and a number of behavioral changes have been reported following developmental exposure to oral doses between 0.01 and 1 mg/kg bw/day (*40, 42, 104, 120-123, 126, 129, 131-133*).

With the exception of a study that showed a slight increase in receptive behavior in females and an impairment of sexual performance in males (*121*), the loss of behavioral sexual dimorphisms do not relate to reproductive behavior (*108, 113, 134*). For instance, responses to novelty and exploratory behavior are sexually dimorphic behaviors where female mice tend to display more of these behaviors than males (*38, 126*). Bisphenol A seems to dampen this sex-difference by reducing the expression of these behaviors in female mice ("defeminization" or "masculinization") exposed during development, either through gestation via the dam with oral doses of 0.01 mg/kg bw/day or through gestation until weaning at 0.04 mg/kg bw/day (*38, 126*).

While a loss of sexual dimorphism seems to be one general trend observed in the behavior literature, findings for other effects can be more difficult to interpret. A number of studies have looked at the relationship between developmental exposure to bisphenol A and increased activity. The studies that most directly support an effect of increased activity administered bisphenol A directly into the brain (*124, 125, 135, 136*). This route of administration limits the ability to interpret these studies in relation to human exposure levels as well as to compare the findings to results from other studies that use more typical routes of administration. Other studies using similar behavior assessments have not reported differences in spontaneous motor activity in the offspring of dams orally treated with a range of doses from 0.1 – 400 mg/kg bw/day (*42, 137*). Indications of increased activity based on other types of behavioral tests are also mixed. Some studies report no impact of bisphenol A treatment on activity (*99, 133, 138*), increased morphine-induced locomotion in animals treated during development with bisphenol A (*127, 139*), no difference between control and bisphenol A treated animals in response to methylphenidate, a drug used to treat attention deficit hyperactivity disorder (ADHD) (*138*), and decreased amphetamine-induced activity in bisphenol A-treated male rats (*38*). The literature provides more consistent support for a loss of sexual dimorphism in locomotor activity. Bisphenol A exposure during development eliminated statistically significant sex differences

observed in control animals where females are more active than males (*113, 116*), or caused significant differences in activity consistent with a loss of sexual dimorphism, i.e., increased activity in male, but not female rats (*140*),

Certain behavioral effects such as alterations in locomotor activity, reward behavior, response to novelty, motivation, cognition, and attention can display some degree of sexual dimorphism but also implicate involvement of the dopaminergic system, a monoaminergic neurotransmitter. Interactions with the dopaminergic system are supported by findings that bisphenol A can alter the gene expression of D1, D3, and D4 dopamine receptors (*128, 136, 141*) and dopamine transporters (*136, 142, 143*). In addition, several studies report that perinatal exposure to bisphenol A can alter (usually decrease) expression of the rate limiting enzyme for dopamine synthesis, tyrosine hydroxylase (TH), that catalyzes the conversion of tyrosine to a pre-cursor of dopamine, dihydroxyphenylalanine (DOPA), in several regions of the brain including the substantia nigra (*136, 144*), the anteroventral periventricular nucleus of the hypothalamus (AVPV) (*115*), midbrain (*142*), limbic area (*143*), and rostral periventricular preoptic area (*116*).

Additional support for the brain as a target of bisphenol A is provided by a number of studies that report neural alterations at the cellular level including interactions with or changes in measures of expression of a number of receptors involved in brain function, such as estrogen receptors ERα and ERβ (*39, 145-147*), gamma-aminobutyric acid type A ($GABA_A$) (*148, 149*), progesterone (*150, 151*), aryl hydrocarbon receptor (AhR), retinoic acid receptor (RAR) alpha, retinoid X receptor (RXR) alpha (*152-154*), and thyroid receptors (*84-88*). Other studies report effects on neuronal migration or organization (*155, 156*), synaptogenesis (*157, 158*), GABA-induced currents (*149*), neuronal cell death (*159*), synaptic plasticity (*160*); thyroid receptor-mediated differentiation of oligodendrocytes (*161*), and reduced proliferation of neural progenitor cells (*162*).

The NTP concurs with the CERHR Expert Panel on Bisphenol A that the results of neurological and behavioral studies of exposures of laboratory animals to bisphenol A during development raise questions about possible risks to human development. The NTP also concurs 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. Overall, the current literature provides a collection of findings that cannot yet be easily interpreted for biological or experimental consistency or for relevance to human health. Part of the interpretive difficulty lies in reconciling findings of different studies that use different experimental designs and different specific behavioral tests to measure the same dimension of behavior.

- Mammary gland

There is evidence from rodent studies suggesting that perinatal exposure to bisphenol A via subcutaneous mini-pump at administered doses of 0.0025 to 1 mg/kg bw/day causes tissue changes ("lesions") in the mammary gland that may signal an increased susceptibility to develop mammary gland tumors later in life (*44, 45*). The evidence is not sufficient to conclude that bisphenol A is a rodent mammary gland carcinogen or that bisphenol A presents a breast cancer hazard to humans.

While bisphenol A has not been shown to cause cellular changes or cancer of the mammary gland in female rats and mice exposed as adults (*163*), two recent studies suggest that exposure of rats to bisphenol A during gestation may lead to the development of lesions in adulthood, ductal hyperplasia and carcinoma *in situ*, that may potentially progress to tumors, i.e., "preneoplastic" lesions (*44, 45*). In the study by Murray *et al.* (*45*) rats were treated with 0.0025 – 1 mg/kg bw/day bisphenol A during pregnancy by subcutaneous mini-pump. Significant increases in the incidence of hyperplastic ducts were reported in all dose groups of female offspring on post-natal day 50 and only in the lowest dose group of 0.0025 mg/kg bw/day on post-natal day 95 (sample sizes range from 4 – 6). A more severe lesion, carcinoma *in situ,* was present in female offspring in the 0.25 and 1 mg/kg bw/day groups on postnatal day 50 (25% incidence for both treatment groups) and postnatal day 95 (33% incidence for both treatment groups). These findings are supported by a study by Durando *et al.* (*44*)[10] where pregnant rats were treated with 0.025 mg/kg bw/day, again using a subcutaneous mini-pump. In this study, the percent of hyperplastic ducts was significantly increased in the female offspring at both postnatal days 110 and 180 (~2 – 5-fold). A non-significant increase in the incidence of ductal carcinoma *in situ* was noted following adult treatment with a subcarcinogenic dose of *N*-nitroso-*N*-methylurea, a chemical used in cancer research to assess susceptability to carcinogens (2/15 compared to 0/10 in control animals).

These findings are generally consistent with other reports of changes in mammary gland growth and development following perinatal exposure to bisphenol A that are related to an altered rate of maturation, e.g., advanced fat pad maturation, delayed lumen formation, enhanced duct growth, adoption of a pregnancy-like state, enhanced responsiveness to secondary estrogenic exposures, and potentially increased susceptibility to carcinogenesis, e.g., increased number or density of terminal end buds and ducts (*44, 45, 164-170*). Overall, these findings have been interpreted as indicating that developmental exposure to bisphenol A causes differential effects on maturation of epithelial and stromal elements in the breast tissue that may lead to a predisposition to disease onset later in life.

With the exception of an oral dosing study conducted by Moral *et al.* (*170*) that reported an increased number of mammary gland terminal ducts in the female offspring of rats treated during gestation with 0.250 mg/kg/day, the cellular and tissue-level effects on the mammary gland occurred following subcutaneous treatment via mini-pump with bisphenol A at doses of 0.000025 to 10 mg/kg/day (*44, 45, 164, 166-169*). The findings most closely linked to an "adverse" outcome, ductal hyperplasia and carcinoma *in situ*, were reported at 0.0025 – 1 mg/kg/day (*44, 45*).

[10] The study by Durando *et al.* (*44*) implied that 99.9% DMSO was used in the mini-pump ["Pumps are designed to deliver 25 BPA (Sigma-Aldrich de Argentina S.A., Buenos Aires, Argentina) or only DMSO (99.9% molecular biology grade, Sigma-Aldrich de Argentina S.A.)"]. The manufacturer of the mini-pump does not recommend use of DMSO concentrations greater than 50% because it can degrade the pump reservoir material and potentially result in tissue inflammation and edema. For this reason, the CERHR Expert Panel on Bisphenol A considered this study critically flawed (*2*). The NTP concurs that use of a high concentration of DMSO is a technical short-coming, but is not convinced that this factor could account for the observed results. The NTP also considered the possibility that potential pump degradation could result in variations in administered dose, but concluded that the study was still useful to consider in the context of other findings.

Certain aspects of mammary gland cancer differ between rats and humans, e.g., metastases are uncommon in rodents, but the lesions identified in these two recent studies, ductal hyperplasia and carcinoma *in situ*, are generally recognized as intermediary steps in chemical-induced mammary gland cancer in the rat and as pre-neoplastic lesions in the human (*171-174*). The appearance of ductal hyperplasia and carcinoma *in situ* are similar enough between rats and humans that these findings in the rat are considered relevant to humans (*172*). In humans, a greater than mild degree of ductal hyperplasia and ductal carcinoma *in situ* are associated with increased relative risk of developing invasive breast carcinoma. It is important to note that the development of these lesions does not guarantee the formation of tumors or cancer in rats or humans and they are most appropriately interpreted as risk factors. If similar changes occur in women, the increased relative risks for developing invasive breast cancer range from 1.5 to 5-fold for moderate and atypical ductal hyperplasia and 8.0 to 10.0-fold for ductal carcinoma *in situ* (*175*). The relative risk is based on a comparison to women of the same age in the general population. For example, a 50-year old woman has a 1 in 39 chance of developing invasive breast cancer in the next 10 years. If a 50-year woman has atypical ductal hyperplasia, a form of ductal hyperplasia associated with a moderate level of increased relative risk (4 to 5-fold), then her chance of developing invasive breast cancer in the next 10 years increases to approximately 1 in 10 to 1 in 8.

The current literature is not sufficient to establish the reproducibility of the ductal lesion findings by multiple independent investigators. Bisphenol A was not shown to induce neoplastic or non-neoplastic lesions in the mammary gland of female rats (~74 and 135 mg/kg bw/day) or mice (650 and 1300 mg/kg bw/day) in two-year dietary cancer bioassays where exposure was initiated in young adult animals (5-weeks of age) (*163*). However, these studies did not include perinatal exposure and the NTP recognizes that adult-only exposure may not be sufficient to detect chemical carcinogens in hormonally-responsive tissues such as the mammary gland (*174*). Most of the toxicology studies of bisphenol A that included assessment of females following developmental exposure either (1) did not report examination of the mammary gland (*29, 35, 111, 176, 177*), or (2) collected mammary gland tissue but did not prepare the tissue in a manner that would readily reveal these changes, i.e., whole mounts (*33, 99*). The limited assessment of the mammary gland in these studies is critical because it is not clear that, if present, intraductal epithelial proliferations would have been detected during the routine histopathologic examinations. While more severe lesions, such as the presence of a mammary mass, would be detected during routine necropsy, the studies by Ema *et al.*, (*99*) and Tyl *et al*., (*33*) were primarily designed to detect effects on reproduction and development and not tumor incidence. Animals were not followed-up for a sufficiently long period of time to necessarily expect to observe tumors in control animals or differences in tumor incidence between treatment groups. In both of these studies, mammary gland tissues in the parental (F0) and F1 generations of females were only examined after weaning of their pups and the animals would have been well under one year of age at the time of tissue collection.

The NTP concurs with recent reviews (*2, 178*) that additional data are needed to more completely understand the possible long-term consequences of disrupting mammary gland development in animals by bisphenol A exposure and its significance for human health. Namely, long-term follow-up studies with sufficient statistical power should be conducted to evaluate if the ductal hyperplasia and carcinoma *in situ* progress to mammary gland tumors, preferably

without the use of a secondary chemical challenge in adulthood. In addition, conducting the appropriate pharmacokinetic studies for the subcutaneous mini-pump would aid in interpreting the results. While researchers predict that circulating levels of total and free bisphenol A in the subcutaneous mini-pump studies would be quite low based on the administered dose (≤ 1 mg/kg bw/day), the lack of supporting pharmacokinetic information limits the ability to make comparisons to human exposures.

- Prostate and Urinary Tract

There is some evidence that perinatal exposure to bisphenol A in rodents may alter prostate and urinary tract development and predispose the prostate to develop hormonally-induced pre-neoplastic lesions later in life. The evidence is not sufficient to conclude that bisphenol A is a rodent prostate gland carcinogen or that bisphenol A presents a prostate cancer hazard to humans.

In mice, exposure of pregnant dams to bisphenol A at an oral dose of 0.010 mg/kg bw/day has been shown in one study to alter prostate development in offspring by increasing the number of prostatic ducts, ductal volume, and the proliferation of a cell population implicated in the development of prostate cancer (basal epithelial cells) in one or more regions of the prostate (*46*). This study also reported a urinary tract deformation where the urethra narrows near the neck of the bladder, an effect that, if permanent, could contribute to urine flow disorders. These effects were observed in fetal mice and it is unclear if they persist into adulthood or relate to a clear adverse health outcome. It is important to note that other studies have not reported severe consequences of urinary tract constriction in adult animals exposed during development that might be predicted based on the finding by Timms *et al*. including bladder stones, hydronephrosis, hydroureter, or other indications of kidney toxicity.

In Sprague-Dawley rats, subcutaneous injection of neonates with 0.010 mg/kg bisphenol A followed by adult hormone treatment[11] was reported to cause 100% of the animals to develop "low" grade (3/10 animals) or "high" grade (7/10 animals) prostate intraepithelial neoplasia (*43*).[12] The incidence of prostate intraepithelial neoplastic lesions in animals that did not receive the adult hormone treatment was not significantly different from controls (2/6 versus 1/9 in control animals). Proposed biological mechnisms to account for the effects of bisphenol A on the prostate include altered DNA methylation patterns in genes that help regulate prostate development and growth as an epigentic mode of action (*43, 180*). The use of adult hormone treatment to promote the development of prostate intraepithelial neoplasia lesions complicates

[11] Animals were given Silastic capsule implants packed with estradiol and testosterone that result in serum concentrations of ~75 pg/ml estradiol and 3 ng/ml testosterone. This hormone treatment is intended to mimic the ratio of estradiol to testosterone in the aging male.

[12] One other study assessed bisphenol A's ability to predispose the prostate to develop prostate intraepithelial neoplasia lesions and tumors (*179*). In this study, female F344 rats were orally dosed with 0.05, 7.5, 30, or 120 mg/kg bw/day of bisphenol A during pregnancy and lactation. In order to induce prostate lesions and tumors, male offspring were treated with a chemical carcinogen, 3,2'-dimethyl-4-aminobiphenyl (DMAB). No statistically significant changes in prostate intraepithelial neoplasia lesions or carcinomas were observed. Differences between this study and the report of Ho *et al*. may be related to age at exposure (fetal versus neonatal and fetal), rat strain (F344 versus Sprague–Dawley), carcinogenic insult (DMAB versus estradiol + testosterone), route of administration (subcutaneous versus oral to dams), or other factor such as animal husbandry and housing.

the interpretation of this study when considering its relevance to human bisphenol A exposure. However, as discussed in more detail below, rodents are normally resistent to developing prostate cancer and the use of hormone treatment, chemical treatment, or other alternative animal model to obtain a more sensitive rodent model is considered an acceptable and recommended strategy in prostate cancer research (*174*).

The findings of Ho *et al.* (*43*) are consistent with a recent report of increased expression of cytokeratin 10 (CK10), a cell-marker associated with squamous differentiation, in adult male offspring of pregnant mice orally treated with 0.020 mg/kg bw/day bisphenol A during gestation (*181*). Chronic exposure to high doses of potent estrogens, such as diethylstilbestrol, leads to squamous metaplasia of the prostate, a tissue change characterized by a multilayering of prostatic basal epithelial cells. Squamous metaplasia is associated with benign prostatic hyperplasia or long-term estrogen treatment in patients with benign or malignant prostatic disease. The induction of CK10 expression in basal epithelial cells is an early indicator of changes leading to estrogen-induced squamous metaplasia. While the long-term health consequences of such an alteration are unclear, prostatic basal epithelial cells are implicated in the initiation and early progression of prostate cancer due to their function in maintaining ductal integrity and regulating the differentiation of luminal epithelial cell differentiation (*182*). It is important to note that prostates in the Ogural *et al.* study appeared morphologically the same as control animals based on the staining technique normally used in pathology (hematoxylin and eosin, or H&E). A stain specific for squamous keratin was required to detect the change. Thus, it is unclear whether similar changes in basal epithelial cell phenotype were present in other studies that evaluated the prostate using only an H&E stain.

The NTP concurs with the CERHR Expert Panel on Bisphenol A and another recent evaluation (*2, 178*) that additional studies are needed to understand the effects of bisphenol A on the development of the prostate gland and urinary tract. Studies should attempt to confirm these findings and include longer periods of follow-up to understand the significance of the structural and cellular effects observed in fetuses and to clarify the relevance of prostate intraepithelial neoplastic lesions resulting from bisphenol A exposure to the development of prostate cancer in these animals. Future research to clarify the role of bisphenol A in the development of prostate cancer presents a scientific challenge. Unlike humans where prostate cancer is common, it is the most common non-skin cancer in American men (*183*), rodents rarely develop prostate cancer. Of the almost 4,550 rats and mice used as controls in NTP 2-year inhalation or feed studies conducted during the last decade, only 1 cancerous tumor and 17 benign tumors ("adenoma") of the prostate gland were detected (*183*). No substances, including bisphenol A (*163*), have been identified as causing prostate tumors in NTP studies (*174*). The NTP has long recognized the limits of the traditional rodent cancer bioassay for detecting chemical-induced prostate tumors and organized a workshop in May 2006 to address this issue (*174*). Suggested strategies to improve the sensitivity of rodent models for detecting prostate cancer included using alternative models, e.g., genetically modified, and/or initiating exposure in perinatal life. In addition, NTP workshop participants suggested a more detailed histopathologic evaluation of the prostate because the assessment of human carcinogenic potential may be better determined based on chemical-induced preneoplastic changes rather than tumor incidence.

During its evaluation of bisphenol A exposure and prostate development, the NTP also considered a number of studies in rats or mice that have detected increased prostate weight at low doses (*107, 184*) or failed to detect this effect (*29, 33, 35, 99, 108, 113, 179, 185-190*). Prostate weight effects have taken on a special significance in the controversy surrounding bisphenol A because elevated prostate weight was the first "low" dose finding reported in laboratory animals (*107*) and prompted numerous follow-up studies. Attempts to understand the basis for discordant findings has generated considerable scientific discussion and debate including their review at the NTP-EPA Low-Dose Peer Review workshop mentioned earlier (*62*). In brief, the NTP believes that the overall conclusions of the Bisphenol A Subpanel of the NTP Low-Dose Peer Review remain valid with respect to "low" dose effects on prostate weight, i.e., increased prostate weight cannot be considered a general or reproducible finding.

More importantly, it is not clear that prostate weight should continue to be considered a critical endpoint in risk evaluations of bisphenol A given the relative crudeness of this measure. Changes in organ weight may be useful to identify potential target tissues, but become less important when additional data relating to structural, cellular, or functional integrity are available. Prostate enlargment does not correlate with the development of prostate histopathology or cancer in rodents, and the evaluation of prostate weight without corresponding assessment of histopathologic changes is not considered useful for determining carcinogenic potential (*191*).

In addition, changes in prostate weight are not necessarily observed in the same bisphenol A studies that report prostatic cellular or tissue-level changes. For example, no effects on prostatic lobe weight were observed in studies that reported (1) increased incidence and susceptibility to develop prostate intraepithelial neoplastic lesions (*43*), (2) changes in the prostatic periductal stroma and decreases in androgen-receptor positive stromal cells and epithelial cells positive for prostatic acid phosphatase (PAS), an enzyme produced by the prostate that can be found in higher amounts in men with prostate cancer (*192*), and (3) increased expression of CK10 in adult mice exposed as fetuses to 0.020 mg/kg bw/day via treatment of the dam or during adulthood to high doses of bisphenol A (2 – 200 mg pellets implanted under the skin for 3-weeks) (*181*).

- Puberty

NTP concurs with the CERHR Expert Panel on Bisphenol A that limited data are available at low doses to suggest an effect of accelerating the onset of puberty in female mice. Early onset of puberty has been observed in offspring of CF-1 mice orally treated with 0.0024 mg/kg/day during gestation (*47*) or C57BL/6 mice orally dosed with 0.2 mg/kg/day during gestation and lactation (*40*). These findings are supported by another study that noted an early onset of puberty in female ICR/Jcl mice whose mothers were treated with 0.02 mg/kg bw/day bisphenol A during gestation by subcutaneous injection (*176*). Two studies reporting effects on mammary gland growth and differentiation in female offpsring of CD-1 mice treated with bisphenol A during pregnancy through a subcutaneous mini-pump are consistent with an impact of bisphenol A on timing of puberty [(*164, 167*), reviewed in (*193*)]. In humans, early onset of puberty in girls is associated with elevated risk of developing breast cancer, early bone age maturation, and psychosocial impacts that include influencing age at first sexual intercourse and increasing risk for certain adolescent risk behaviors (*194-196*). Depending on the magnitude of the finding, early onset of puberty in laboratory animals can be considered an "adverse" effect in

reproductive toxicology risk assessment (*194*). The magnitude of the acceleration in puberty reported in the mouse studies ranges from 1 to 4.5 days (*40, 47, 176*).

Other studies have reported no effects on the timing of puberty in female mice [CF-1(*185*) or CD-1 (*33, 165*)] whose dams were treated with "low" doses of bisphenol A delivered orally or by subcutaneous mini-pump during gestation or during gestation and lactation. It is unclear if the inability of these studies to reproduce the advanced onset of puberty finding was due to variations in mouse strain and stock, timing of exposure, diet, or other facets of experimental design. The most consistent difference between the "positive" and "negative" studies lies in the approach used to measure onset of puberty. Age at first estrus is the most accurate indicator of puberty in rodents. This occurs at the same time as vaginal opening in rats. However, in mice, vaginal opening does not correlate well with puberty and the first day of detecting cornified cells in a vaginal smear, a sign of first estrus, is used to indicate the onset of puberty (*197*). The studies by Ashby *et al*., Markey, *et al.*, and Tyl *et al.*, (*33, 165, 185*) that did not detect an effect of bisphenol A relied on age at vaginal opening in mice rather than the use of vaginal smears to assess onset of puberty. An additional issue associated with interpreting the study by Ashby *et al*., (*185*) is the finding of a significant 3.6 day delay in the age of vaginal opening in the diethylstilbestrol positive control group (0.0002 mg/kg bw/day) when compared to the vehicle control group. A delay in puberty is inconsistent with the predicted estrogenic effect of accelerated puberty in the diethylstilbestrol group.

Additional studies are needed to establish the reproducibility of the finding that bisphenol A causes early onset of puberty in female mice at very low doses. The study by Howdeshell *et al.*, (*47*) reported a ~ 2.5 day acceleration of puberty in female offspring of mice orally treated with 0.0024 mg/kg bw/day during pregnancy based on a measure that is not standard in toxicology (the interval between vaginal opening and first estrus). Using the more standard interval of days from birth to first estrus, Ryan *et al*. (*40*) found ~ 4.5 day acceleration in puberty in the female offspring of dams treated during gestation and lactation with an oral dose of 0.2 mg/kg bw/day, but no effect at 0.02 mg/kg bw/day. The study by Honma *et al*. (*176*) reported a ~1 day earlier onset of puberty in the offspring of mice treated with 0.02 mg/kg bw/day by subcutaneous injection during pregnancy. As discussed previously, doses delivered orally and by subcutaneous injection in adult animals, including pregnant dams, cannot be directly compared due to route of administration differences in the metabolism of bisphenol A.

The data in female rats are less compelling for a possible "low" dose effect on puberty. A finding of accelerated puberty has been reported in Wistar rats (*44*), but most of the "low" dose literature does not support an effect (*29, 35, 45, 99, 113, 198, 199*).

The effects of bisphenol A on puberty in rats at "high" doses are generally inconsistent with the "low" dose effects reported in the mouse studies by Howdeshell *et al.* (*47*), Ryan *et al.* (*40*), and Honma *et al.* (*176*). Only one study has reported an effect on puberty in the predicted direction, i.e., acceleration following subcutaneous treatment on postnatal days 0 to 9 (*111*). Other studies reported no effect (*108-110*) or a delay in puberty at ≥ 50 mg/kg bw/day (*29, 35*). Four of these studies used a positive control group (*35, 108, 110, 111*). In these studies, responses to potent estrogens based on age at vaginal opening ranged from no effect (*108*), to statistically significant small or moderate acceleration [1.7 days (*35*); 2.4 days (*111*); 3.6 days (*110*)].

An area of uncertainty in the assessment of puberty is reconciling the general absence of an effect at "low" doses in rats with the mouse studies that found early onset of puberty in females when puberty was assessed by age at first estrous. The differences in outcomes cannot be attributed to use of single insensitive strain or stock as a variety of rat models were used in the "negative" studies: Sprague-Dawley, Wistar, Wistar-Furth rats, Wistar-derived Alderley Park, CD, and Donryu. Moreover, three of the "negative" puberty studies reported other "low" dose effects (*45, 113, 198*). Based on an evaluation of two negative studies that included "low" dose treatment groups and that used a positive control compound (*35, 113*), there is some support for a conclusion that vaginal opening may not be a sensitive indicator of estrogenic response in all strains of rat or experimental designs. The study by Tinwell *et al.* (*35*) reported a relatively small acceleration in puberty, 1.7 days, in Wistar-derived Alderley Park rats treated with what is considered a high dose level of ethinyl estradiol (0.2/0.1 mg/kg bw/day orally to dams during pregnancy). In contrast, the study by Kubo *et al.* (*113*) reported a more profound acceleration in puberty of 5.9 days in female offspring of Wistar rats exposed to diethylstilbestrol (0.050 mg/L in drinking water) during pregnancy and lactation (*113*). Another observation made from the rat studies that used a positive control group is that larger impacts on puberty onset (> 3 days) were more likely to be observed in studies that exposed animals during gestation and lactation or lactation (*110, 111, 113*) compared to gestation only (*35*); although, the Kwon *et al*. study (*108*) does not fit this profile (no effect on puberty following oral treatment with 3.2 – 320 mg/kg/day during gestation and lactation).

In summary, additional research is needed to assess the robustness of altered puberty at dose levels in the very low μg/kg bw/day range in mice, i.e. 0.0024 mg/kg bw/day. Research directed towards understanding the apparent differences in response between rats and mice on this measure would also be valuable. This issue has implications not just for the evaluation of bisphenol A, but also for characterizing possible effects on puberty for other weakly estrogenic compounds.

- Other Effects Considered

A variety of other effects in laboratory animals have been linked to "low" dose bisphenol A exposure during development, including decreased sperm quantity or quality, obesity, disruption of meiosis, changes in reproductive hormone levels, or cellular effects in reproductive tissues. These effects had less impact in shaping NTP's conclusions on potential risks to humans from bisphenol A exposure than the developmental effects observed at "high" doses on survival and growth and the "low" dose effects on brain and behavior, mammary gland, prostate gland, and onset of puberty in females described above.

In some cases, the relationship between a specific cellular- or tissue-level finding and a potential health effect in the whole organism is unclear. This is because there is often uncertainty about the functional impact of a cellular or mechanistic finding, such as the altered level of a receptor protein or change in enzyme activity. For example, the potential health impact that may result from uterine changes characterized by altered ERα and ERβ expression and from an increase in the number and appearance of uterine epithelial cells is unclear (*200*).

In other cases, the literature is not sufficiently developed. Newbold *et al.* (*201*) recently described a number of morphological changes in the ovaries and uteri of 18-month old mice that had received subcutaneous injections of bisphenol A at doses of 10, 100, or 1000 μg/kg on days 1-5 of life. Increases in cystic ovaries and cystic endometrial hyperplasia were statistically significant in the 100 μg/kg dose group but not at 1000 μg/kg. Non-statistically significant increases in the incidence of a variety of other ovarian and uterine proliferative lesions and cysts were also reported. Replication of these findings and further study of the linkage of early and late occurring events will be important in establishing a better understanding of any long-term consequences of exposures of the developing organism to bisphenol A.

As mentioned earlier, NTP Briefs are not meant to serve as comprehensive reviews of the scientific literature. Only key study findings and issues that relate to NTP conclusions on concerns for potential reproductive and developmental health effects in humans are typically presented. However, three reported "low" dose health effects (obesity, decreased sperm count or quality, and abnormalities of meiosis) that ultimately had less impact in determining the NTP's conclusions are briefly discussed below in order to illustrate the interpretive challenges associated with this literature. Two of these examples, obesity and impacts on sperm, are used to demonstrate findings that are not reported consistently enough to be considered reproducible. The third example relates to abnormalities of meiosis and is presented to demonstrate that effects predicted from *in vitro* studies are not necessarily observed in the *in vivo* studies.

Obesity

There is currently insufficient evidence to conclude that bisphenol A exposure during development predisposes laboratory animals to develop obesity or metabolic diseases such as diabetes, later in life. Obesity and metabolic disruption has become a research focus for bisphenol A based on several reports of increased postnatal growth following "low" dose exposure during development and several *in vitro* and *in vivo* studies that report effects related to altered carbohydrate and lipid regulation.

The NTP concurs with the CERHR Expert Panel on Bisphenol A that the effects of bisphenol A on body weight at "low" doses are inconsistent (*2*). A number of studies in rats and mice report increases in post-natal growth following developmental exposure to bisphenol A at oral doses of 0.0024 – 1.2 mg/kg bw/day (*47, 146, 198, 202*) or a subcutaneous dose of 0.5 mg/kg bw/day (*166*). Other "low" dose (≤ 5 mg/kg bw/day) studies in rats and mice have either not detected any significant effect on body weight (*35, 40, 42, 44, 99, 108, 113, 189, 201, 203*) or reported growth reductions (*29, 107, 137, 176, 204*). Differences in study outcomes cannot easily be attributed to the use of a potentially insensitive rodent model or experimental protocol because several studies that did not detect any significant difference in body weight reported other effects at "low" dose levels (*40, 42, 44, 113, 204*). The bases for the inconsistent findings are unclear but may relate to factors such as diet and differences in experimental design or analysis.

The data are currently too limited to conclude that developmental exposure to bisphenol A causes diabetes or other metabolic disorders later in life. Two studies in laboratory animals have assessed endpoints related to carbohydrate or lipid regulation. In adult male mice, a single subcutaneous dose of 0.010 or 0.100 mg/kg bw/day bisphenol A caused decreased blood glucose

and increased plasma insulin (*205*). Additionally, increased pancreatic insulin content and insulin resistance was reported at 0.100 mg/kg bw/day (administered orally or by subcutaneous injection) after a slightly longer period of dosing (4-days) (*205*). A recent study by Miyawaki *et al.* (*202*) assessed a variety of endpoints related to carbohydrate and lipid regulation in 1-month old mice that were exposed through maternal treatment during gestation and lactation with 0.001 or 0.010 μg/ml bisphenol A in drinking water (~0.26 and 2.42 mg/kg bw/during gestation). Endpoints included body weight, adipose tissue weight, and blood concentrations of leptin, total cholesterol, triglycerides, non-esterified fatty acid and glucose. Body weight and total cholesterol were significantly increased in female offspring in both dose groups although adipose tissue weight and leptin levels were only significantly increased in the 1 μg/ml treatment group. Male offspring in the high dose of 10 μg/ml were significantly heavier and had increased adipose tissue weight. Leptin levels were not associated with either of these effects in males. Significantly increased triglycerides and non-esterified fatty acid and decreased glucose were observed in male offspring in the low dose group of 1 μg/m. Although this study addresses the hypothesis that developmental exposure to bisphenol A can affect carbohydrate and lipid metabolism in postnatal life, the inconsistent pattern of effects on serum lipid levels, leptin, and glucose and lack of control for litter effects[13] makes the study on its own insufficient to draw any conclusion.

More research in this area is warranted. Several *in vitro* studies report effects of bisphenol A related to carbohydrate and lipid regulation including effects on pancreatic cells that govern the release of insulin (β-cells) and glucagon (α-cells), altered differentiation of fibroblast cells into adipocytes, and altered glucose transport in adipocytes (*206-210*). Some of the effects on pancreatic cells are very rapid, e.g., altered frequency of glucose-induced calcium oscillations in α- and β-cells, activation of cAMP response element binding protein, and appear to be mediated by ncmER (*65, 67, 211*). Effects mediated through the ncmER are of interest because bisphenol A has been shown to activate this receptor *in vitro* at a concentration of 1 nM, which is similar to the active concentration of diethylstilbestrol (*65, 67*).

Decreased Sperm Count and Sperm Quality

There is currently insufficient evidence to conclude that bisphenol A exposure during development or adulthood causes decreased sperm count or sperm quality. A large number of studies have addressed this issue but the literature is inconsistent and not easily reconciled.

- Exposure during development

There are some indications that treatment with "high" oral doses of bisphenol A during development or young adulthood can impact sperm quantity in laboratory rats (*29, 34, 35*). Tan *et al.* (*34*) reported that 33% of rats did not show any evidence of having a spermatogenic cycle after treatment in young adulthood with 100 mg/kg bw/day of bisphenol A. Other reported decreases in measures of testicular or epididymal sperm count and sperm production were more modest and ranged from 10 to 19% at doses of 50 and 500 mg/kg bw/day (*29, 35*). In addition, in

[13] 16 -25 males or females were reported for each treatment group however these animals were derived from only 3 litters per treatment group (i.e., the effective sample size is three instead of 16-25) (*202*).

the three generation rat study conducted by Tyl *et al*. (*29*), significant decreases in sperm parameters were only observed in certain generations of similarly exposed males in the high dose group of 500 mg/kg bw/day: ~18% decrease in epididymal sperm concentration in F1 males; ~19% decrease in testicular daily sperm production in F3 males and no significant effects in the F0 or F2 generations. Testicular or epididymal histopathology was not detected in any treatment group (*29*). Significantly decreased sperm motility and an increased percentage of abnormal sperm has also been reported following "high" dose subcutaneous injection, ~25 mg/kg bw/day[14], to neonatal mice in a study conducted by Aikawa *et al.* (*212*). Again, these effects were not associated with testicular histological alterations.

Effects on sperm parameters have been reported at lower doses administered orally or by subcutaneous injection.[15] vom Saal *et al*. (*213*) reported a ~19% decrease in testicular daily sperm production in adult male mice exposed to bisphenol A as fetuses via maternal dosing with 0.02 mg/kg bw/day (higher dose levels were not tested). Toyama *et al.* (*214*) observed increased incidences of several measures of abnormal sperm morphology (40 – 80% compared to < 0.3% in controls) in mice treated with > 0.17 mg/kg or rats treated with > 0.33 mg/kg by subcutaneous injection[16] of bisphenol A every other day during post-natal days 2 to 12.

However, a number of larger studies have not reported effects on sperm parameters following exposure during development at "high" or "low" dose levels (0.0002 – 600 mg/kg bw/day) (*33, 99, 188-190, 215*).

- Exposure during adulthood only

Several studies have reported effects on sperm parameters in mice or rats exposed to "low" doses of bisphenol A only during adulthood. In rats, these effects are reported following oral dosing of 0.02 – 200 mg/kg bw/day for six days (~24 – 32% decreased daily sperm production per gram tissue) (*216*), 0.0002 – 0.02 mg/kg bw/day for 45 days (~23–41% decrease in epididymal sperm motility; ~18-27% decrease in epidiymal sperm count at 0.002 – 0.02 mg/kg bw/day) (*217*), and 0.0002 – 0.02 mg/kg bw/day for 60 days (~30–45% decrease in epididymal sperm motility; ~12-40% decrease in epidiymal sperm count at 0.002 – 0.02 mg/kg bw/day) (*218*). In adult mice, "low" dose effects on sperm are observed at oral doses of 0.025 – 0.1 mg/kg bw/day for 30 days (~16 – 37% decrease in weight corrected testicular or epididymal sperm count)(*100*) and subcutaneous dosing with 0.02 and 0.2 mg/kg bw/day for 6 days (abnormal sperm morphology) (*219*).

Other larger studies have not reported effects in adult animals at these doses. The 2-generation mouse study conducted by Tyl *et al*. (*33*) reported a 15% decrease in epididymal sperm

[14] Administered dose was 0.050 mg/pup. This is approximately equal to 25 mg/kg/day assuming that a neonatal mouse weighs 0.002 kg.

[15] Talsness *et al*. (*204*) reported effects on sperm quantity in rats exposed during gestation to 0.1 and 50 mg/kg bw/day but this study is not included in the discussion because (1) reported effects included an increase in sperm number which was opposite the effect observed in the positive control group, and (2) effects on daily sperm production appeared inconsistent over time and across dose.

[16] Administered doses were ≥ 0.001 mg/pup in the mouse and ≥ 0.01 mg/pup in the rat. These doses are approximately equal to 0.17 to 0.5 mg/kg in the mouse and 0.33 - 1.33 mg/kg in the rat assuming that body weight between post-natal days 2 to 12 ranges from 0.002 to 0.006 kg in the mouse and 0.0075 and 0.03 kg in the rat.

concentration in F0 generation animals at the highest dose tested of 600 mg/kg bw/day but not at lower doses of 0.003 to 50 mg/kg bw/day. Ema *et al.* (*99*) also did not detect an effect on sperm measures in the F0 generation in a rat multigeneration study at oral doses of 0.0002 to 0.2 mg/kg bw/day. The finding by Sakaue *et al.* (*216*) of a ~24 – 32% decrease in sperm production in adult Sprague-Dawley rats (obtained from CLEA Japan, Inc.) was not reproduced in a study using larger sample sizes of Sprague-Dawley rats obtained from a Charles River UK (*220*).

The basis for the inconsistent findings is not clear. One proposed explanation is that rodent species, strains, and breeding stocks differ in their responsiveness to estrogens (*51*). Species and strain differences in response to estrogen have been documented, but animal model sensitivity varies depending upon the specific trait being assessed [discussed in (*2, 51, 189*)]. Studies that include sperm assessment in the bisphenol A literature are too varied in terms of periods of dosing, use of positive control, e.g., none used, ethinyl estradiol, or 17β estradiol, and other aspects of experimental conduct to determine if differences in sensitivity of the animal model used can account for the inconsistent findings on sperm quantity and quality.

Chromosome and Meiosis Abnormalities

Disruption of the processes that distribute chromosomes during meiosis or mitosis can result in aneuploid cells, i.e., germ cells that have more or fewer chromosomes than the normal haploid number or somatic cells that have more or fewer chromosomes than the normal diploid number. When this happens in eggs or sperm of humans, it can lead to such conditions as Down Syndrome in which the fetus ends up with 3 copies of chromosome 21, rather than two copies, or a range of syndromes associated with abnormal numbers of sex chromosomes (normal is XX for females, XY for males) such as Klinefelter Syndrome (XXY males) or Turner Syndrome (XO females). If a chemical exposure is capable of inducing aneuploid eggs or sperm, affected individuals would be expected to exhibit problems in achieving or maintaining pregnancy, or to produce aneuploid offspring. While the body of evidence from both *in vitro* and *in vivo* studies provides evidence that bisphenol A can disrupt certain aspects of cell division involving both mitotic and meiotic processes, breeding studies in laboratory animals exposed to bisphenol A do not present results consistent with such effects. Thus, the significance of the reported effects on meiosis and mitosis for mammalian reproduction is not yet clear.

Two *in vivo* studies (*221, 222*) reported that short-term oral exposure to low doses of bisphenol A (≥ 0.020 mg/kg bw/day) in peripubertal or pregnant mice can interfere with meiotic divisions in development of female germ cells ("egg" or "oocyte"). An increase in hyperploid (aneuploid) metaphase II oocytes was observed following treatment with 0.020 mg/kg bw/day. There was not a significant increase in aneuploid embryos. Two subsequent *in vivo* studies (*223, 224*) attempted to replicate these findings. Consistent with the previous findings, they detected no significant effects of bisphenol A exposure on the frequency of aneuploidy in "zygotes" (fertilized oocytes) produced from female mice treated before puberty or as adults with a similar range of doses. In addition, Eichenlaub-Ritter *et al.* (*223*) found no effects of bisphenol A exposure on aneuploid oocytes and Pacchierotti *et al.* (*224*) found no increase in aneuploid or diploid sperm following exposure of male mice to bisphenol A.

A number of *in vitro* studies using cultured mammalian somatic cells have also looked at the potential for bisphenol A to cause aneuploidy. Earlier studies (*225-227*) consistently reported the induction of aneuploidy in various cell lines including SHE, V79, and MCL-5 at concentrations of bisphenol A between 50 and 200 μM (14.4 and 57.6 μg/ml). Recent *in vitro* studies reported effects of bisphenol A on maturation, but not induction of aneuploidy, in mouse oocytes (*223, 228*) or cultured mammalian somatic cells (*229, 230*), increased frequency of mitotic cells with aberrant spindles (*230*), and various effects on cellular and nuclear division in fertilized sea urchin eggs (*231*). Although these new studies provide further evidence of bisphenol A's effects on meiotic and mitotic cell division using a variety of *in vitro* systems and treatment concentrations, no impact of such effects on reproduction is reported in animal breeding studies and the significance of these findings with regard to human health hazards is not clear. If aneuploid eggs or sperm were induced by bisphenol A, it would be expected to result in reduced litter sizes following exposure of one or both parents to bisphenol A. Such an effect is not seen in reproductive toxicity studies of bisphenol A in rats or mice except at very high exposure levels (500 mg/kg bw/day or higher) where other types of toxicities are manifest (*29, 32, 33*), including in the F2 generation (*29, 33*). Findings of significantly decreased litter size or pregnancy loss are reported occasionally at lower doses of bisphenol A (*106, 232*), but in general, most "low" dose studies do not report this outcome including a number of those that report other effects of bisphenol A exposure (*36, 40, 44, 45, 107, 116, 176*).

Are Current Exposures to Bisphenol A High Enough to Cause Concern?

Possibly. The "high" dose effects of bisphenol A in laboratory animals that provide *clear evidence* for adverse effects on development, i.e., reduced survival, birth weight, and growth of offspring early in life, and delayed puberty in female rats and male rats and mice, are observed at levels of exposure that far exceed those encountered by humans. However, estimated exposures in pregnant women and fetuses, infants, and children are similar to levels of bisphenol A associated with several "low" dose laboratory animal findings of effects on the brain and behavior, prostate and mammary gland development, and early onset of puberty in females. When considered together, these findings provide *limited evidence* that bisphenol A has adverse effects on development (**Figure 2b**).

Exposures in humans and laboratory animals can be compared using approaches based on either estimated daily intake (based on aggregating sources of exposure or back calculating from biomonitoring data) or measured blood concentrations of free bisphenol A. Each approach has a unique set of assumptions and limitations. The conclusion of similarities between exposures of certain human populations and laboratory animals treated with "low" doses of bisphenol A is supported by multiple approaches. For this reason, the possibility that human development may be altered by bisphenol A at current exposure levels cannot be dismissed.

Supporting Evidence

A considerable amount of research has been directed towards understanding the levels of human exposure to bisphenol A, either by estimating daily intake or by measuring bisphenol A concentrations in human blood, urine, breast milk, or other tissue. An overarching issue relevant to the bisphenol A biomonitoring studies in both humans and laboratory animals is the accuracy

of the laboratory methods used to measure the compound (see Appendix 1). There is concern that measurements of bisphenol A, especially free bisphenol A, may be too high due to problems related to sample preparation or storage and the analytical technique employed [reviewed in (*2, 11*)]. The NTP recognizes the possibility that the published values of free bisphenol A may, in some cases, not accurately represent the "true" concentrations of free bisphenol A in the blood or body fluids of humans or laboratory animals. However, because of the similarity among values reported with different analytical methods, with the exception of studies that use an enzyme-linked immunosorbent assay (ELISA), the NTP accepts the published values as sufficiently reliable for use in this evaluation.

Daily Intake Exposure Estimates

The vast majority of bisphenol A exposure is through the diet, estimated at ~ 99% (*1*); therefore, estimates of daily intake in humans can be compared to oral doses used in laboratory animal studies where effects considered relevant to human health were observed. Estimates of daily intake are derived using two general approaches. Researchers can use information on the amount of bisphenol A detected in various sources of exposure (i.e., food, food packaging, air, water, dust, etc.) and sum, or aggregate, the measurements to estimate a total daily intake ("aggregating sources of exposure" method). Alternatively, biomonitoring information, such as the concentration of bisphenol A in urine, can be used to estimate, or "back calculate", a total intake that reflects all sources of exposure, both known and unknown. Both approaches for estimating daily intake rely on various assumptions and default values such as average body weight, amount of food or beverage consumed, daily volume of urine output, or ability of a single measurement to characterize exposure.

- Infants and children less than 6 years of age

For infants and children less than 6 years of age, estimates of daily intake were based on aggregating sources of exposure (Table 1). No biomonitoring data, i.e., blood or urine concentration of bisphenol A, are available for these lifestages [reviewed in (*2*)]. An estimated daily intake of ~ 1 μg/kg bw/day for both breast-fed and formula-fed infants was calculated by the CERHR Expert Panel for Bisphenol A (*2*). Higher "worst case" daily intake estimates of 11 - 13 μg/kg bw/day during the first year of life have been calculated for infants (*17*). In children 1.5 to 6 years of age, the range of estimated daily intakes based on aggregating sources of exposure is 0.043 – 14.7 μg/kg bw/day, with 14.7 μg/kg bw/day representing a worst case scenario (*19, 21*).

Although biomonitoring data are not available for infants and children less than 6 years of age, blood and urine levels of free bisphenol A are predicted to be higher in these age groups compared to pregnant women or other adult populations. This is based on information related to age-specific differences in daily intake of bisphenol A and in the ability to metabolize the chemical. More specifically, it is based on observations of (1) higher urinary measurements of total bisphenol A in children (6 - 11 years of age) compared to adolescents and adults (*6*), (2) higher estimated daily intakes of bisphenol A for infants and children (*2, 17, 19*) compared to estimated daily intakes for adults (*2, 17, 27*), and (3) predicted higher blood concentrations of free bisphenol A in infants compared to adults at a given daily intake level based on less efficient

metabolism of bisphenol A in rat fetuses and neonates (*12-14*), and very low or absent activities in human fetuses and premature or full-term infants of the isozymes that govern glucuronidation (*233-235*).

- Adults and children aged 6 years and above

Daily intake estimates for adults and children aged 6 years and older are based on (1) back calculations from the most recent Center for Disease Control and Prevention NHANES data on urinary concentrations of total bisphenol and (2) aggregating sources of exposure (Table 1 and Table 3). Of these estimates, the NTP has more confidence in the estimates based on back calculating from urinary biomonitoring data because all sources of exposure are integrated into the fluid measurement and thus do not have to be identified in advance. However, it is worth noting that the estimates for non-occupationally exposed adults based on aggregating sources of exposure encompass the range estimated from back calculating from urine [aggregating sources of exposure: 0.008 – 1.5 μg/kg bw/day (Table 1); and back calculating based on urine: 0.233 – 0.289 μg/kg bw/day for various categories of adults ages 20+ at the 95th percentile (*27*)]. Fewer studies have estimated daily intakes for children older than 6 years of age and adolescents. In Japanese children and adolescents between the ages of 7 and 19 years, the range of estimated daily intakes based on aggregating sources of exposure is 0.36 to 0.55 μg/kg bw/day (*22*), which is only slightly higher than the estimated range of daily intakes for American children and adolescents based on back calculating from urinary concentration of total bisphenol A [0.311 – 0.348 μg/kg bw/day for children ages 6-11 and 12-19 at the 95th percentile (*27*)].

- Estimated daily intake based on blood biomonitoring

The NTP also considered the appropriateness of estimating daily intake based on back calculations from free bisphenol A measured in human blood and concluded that the scientific uncertainties are currently too large to support this exercise (see Appendix 1). In brief, estimated daily intakes in adults based on this approach are much greater (~500 μg/kg – 1.54 mg/kg bw/day for a 65 kg human) (*3, 236*) than estimates of daily intake based on aggregating routes of exposure (0.008 – 1.5 μg/kg bw/day) (*17, 23*) or from back calculating from urinary data (adults aged 20 – 60+: medians 0.0563 – 0.0334 μg/kg bw/day; 95th percentiles 0.289 – 0.233) (*27*). In addition, data from an intentional dosing study conducted by Tsukioka *et al.* (*237*)[17] provides further support for daily intakes in humans of < 1 μg/kg. Several explanations have been proposed to account for the discrepancy between estimated intake based on blood and urine but they are not sufficient to fully explain it.

Exposure Comparisons Based on Daily Intake

The "high" dose effects of bisphenol A that represent *clear evidence* for adverse effects on development, i.e., reduced survival (≥ 500 mg/kg bw/day) (*28-32*), reduced birth weight and

[17] Tsukioka *et al*. (*237*) used GC/MS with trimethylsilyation (TMS) derivatization (LOQ 0.1 mg/L). Brock *et al.* (*238*) report that use of TMS may produce interfering peaks in the chromatogram. Sample work-up included glucuronidase treatment, solvent extraction, and solid phase clean-up. Few details were presented in the Tsukioka *et al*. (*237*) study on sample preparation process, such as storage temperature.

growth of offspring early in life (≥ 300 mg/kg bw/day) (*28-31, 33*), and delayed puberty in female rats and male rats and mice (≥ 50 mg/kg bw/day) (*29, 33-35*), are observed at dose levels that are more than 3,500-times higher than "worst case" daily intakes of bisphenol A in infants and children less than 6 years of age (≥ 50 mg/kg bw/day versus 0.008 - 0.0147 mg/kg bw/day). The differences in exposures are much greater, more than 160,000-times different, when the high oral dose level is compared to estimated daily intakes for children ages 6-11 and adult women (as an indicator of exposure for pregnant women) at the 95th percentile of 0.311 and 0.271 μg/kg bw/day, respectively (*27*).

However, a number of "low" dose developmental effects have been reported in mice treated orally with bisphenol A including effects on behavior (≥10 μg/kg bw/day) (*36-42*), prostate gland and urinary tract development (10 μg/kg bw/day) (*46*), and early onset of puberty (2.4 and 200 μg/kg bw/day) (*40, 47*). In addition, subcutaneous injection with 10 μg/kg bw/day of bisphenol A during neonatal life in rats results in development of hormonally induced preneoplastic lesions in the prostate later in life (*43*).[18] This non-oral study is considered relevant for comparing exposures because, as discussed previously, the differences in the rate of bisphenol A metabolism seen in adult rats based on route of administration (oral versus non-oral) appear to be greatly reduced in neonatal rats and mice (*12, 58*). As stated earlier, these findings, when considered together, provide *limited evidence* for adverse effects of bisphenol A exposure on development in laboratory animals (**Figure 2b**).

In infants, the doses of 2.4 and 10 μg/kg bw/day are 2.4 - 10 times higher than the estimated daily intake of ~ 1 μg/kg bw/day calculated by the CERHR Expert Panel for Bisphenol A (*2*). Higher "worst case" daily intakes have been calculated for infants by the European Food Safety Authority of 11 - 13 μg/kg during the first year of life (*17*). To the extent these estimates are accurate, then dose levels of 2.4 and 10 μg/kg bw/day slightly exceed (1.1 to 5.4-times) worst case estimates. The doses of 2.4 and 10 μg/kg bw/day are approximately 7.7-32 and 8.9-37 times higher than the estimated daily intakes of 0.311 μg/kg bw/day for children (ages 6-11 years) and 0.271 μg/kg bw/day for adult women at the 95th percentile (*27*).

Exposure Comparisons Based on Blood Concentrations of Free Bisphenol A

No studies in laboratory animals have measured circulating levels of free bisphenol A in the blood following a dosing schedule that mimics human exposures, i.e., long-term dietary low-dose exposure occurring numerous times during the day. However, a number of studies have detected quantifiable levels of free bisphenol A in the blood of adult rodents following a single oral administration of bisphenol A, typically at doses considered high when compared to estimated human daily intakes (500 – 1,000,000 μg/kg for rodents versus < 14.7 μg/kg bw/day for humans) (*3, 19, 27, 236*). These studies were used by Vandenberg *et al.* (*3*) to estimate circulating blood levels of free bisphenol A in rodents at a lower oral dose of 50 μg/kg based on

[18] Preneoplastic lesions in the mammary gland, i.e., ductal hyperplasia and carcinoma *in situ*, have been reported in rats treated as fetuses with 2.5 μg/kg bw/day via a subcutaneous pump implanted in the dam (*44, 45*); however, as discussed previously, studies that administer bisphenol A via subcutaneous pump are considered informative for identifying potential biological effects of bisphenol A, but not for quantitatively comparing exposures in laboratory animals and humans.

the assumption of linear proportionality between administered dose and circulating concentration of free bisphenol A. The estimated peak blood levels of free bisphenol A in the first 30-minutes after dosing at 50 μg/kg ranged from 0.01 to 1.14 μg /L (median 0.11 μg /L) (*3*). Based on this estimate, peak concentrations of free bisphenol A in mice or rats treated with 2.4 or 10 μg/kg bw/day of bisphenol A are projected to be lower than the free blood concentrations measured in humans, including pregnant women (*10, 239*). See Appendix 1 for further details on these calculations.

NTP Conclusions

The NTP concurs with the conclusion of the CERHR Expert Panel on Bisphenol A that there is *some* concern for neural and behavioral effects in fetuses, infants, and children at current human exposures. The NTP also has *some* concern for bisphenol A exposure in these populations based on effects in the prostate gland, mammary gland, and an earlier age for puberty in females.

The scientific evidence that supports a conclusion of *some* concern for exposures in fetuses, infants, and children comes from a number of laboratory animal studies reporting that "low" level exposure to bisphenol A during development can cause changes in behavior and the brain, prostate gland, mammary gland, and the age at which females attain puberty. These studies only provide limited evidence for adverse effects on development and more research is needed to better understand their implications for human health. However, because these effects in animals occur at bisphenol A exposure levels similar to those experienced by humans, the possibility that bisphenol A may alter human development cannot be dismissed.

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.

In laboratory animals, exposure to very high levels of bisphenol A during pregnancy can cause fetal death and reduced birth weight and growth during infancy. These studies provide clear evidence for adverse effects on development, but occur at exposure levels far in excess of those experienced by humans. Two recent human studies have not associated bisphenol A exposure in pregnant women with decreased birth weight or several other measures of birth outcome. Results from several animal studies provide evidence that bisphenol A does not cause birth defects such as cleft palette, skeletal malformations, or grossly abnormal organs.

The NTP concurs with the conclusion of the CERHR Expert Panel on Bisphenol A that there is *negligible* concern that exposure to bisphenol A causes reproductive effects in non-occupationally exposed adults and *minimal* concern for workers exposed to higher levels in occupational settings.

Data from studies in humans are not sufficient to determine if bisphenol A adversely affects reproduction when exposure occurs during adulthood. A number of studies, when considered together, suggest a possible effect on reproductive hormones, especially in men exposed to higher levels of bisphenol A in the workplace. Laboratory studies in adult animals show adverse effects on fertility, estrous cycling, and the testes at exposure levels far in excess of those experienced by humans. A number of other effects, such as decreased sperm counts, are reported for the reproductive system at lower doses in animals exposed only during adulthood, but these effects have not been shown to be reproducible. Laboratory animal studies consistently report that bisphenol A does not affect fertility.

These conclusions are based on information available at the time this brief was prepared. As new information on toxicity and exposure accumulates, it may form the

basis for either lowering or raising the levels of concern expressed in the conclusions.

Figures

Figure 2a. The weight of evidence that bisphenol A causes adverse developmental or reproductive effects in humans.

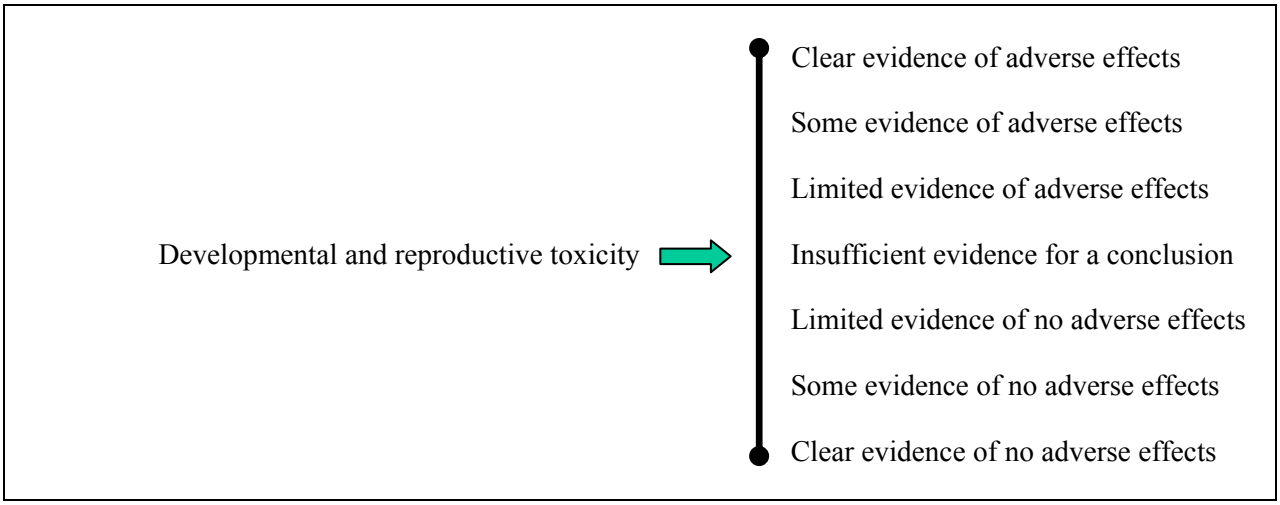
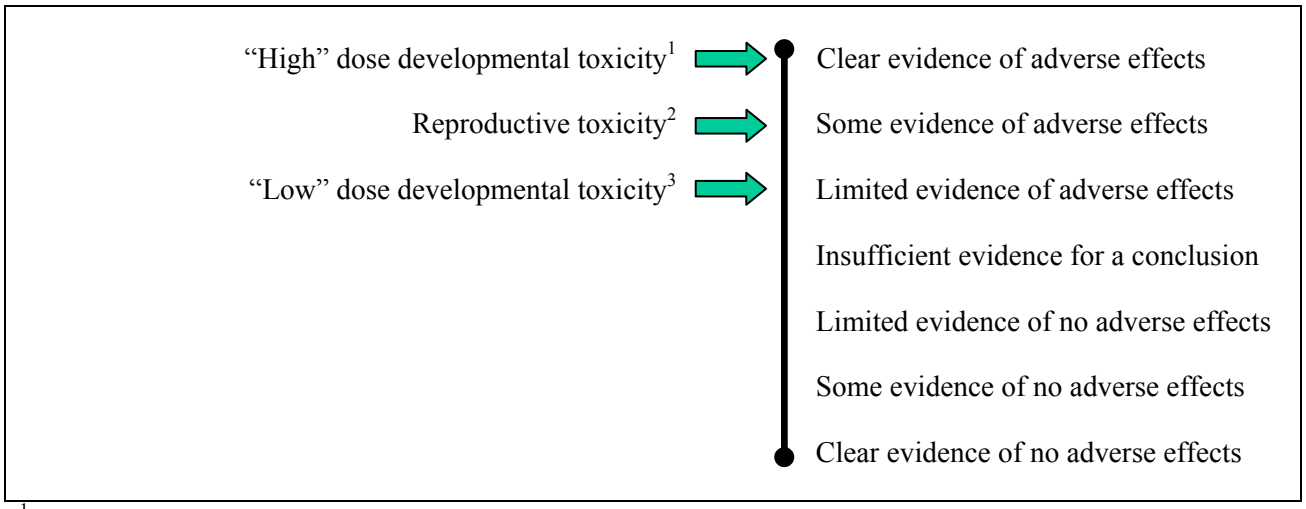


Figure 2b. The weight of evidence that bisphenol A causes adverse developmental or reproductive effects in laboratory animals.

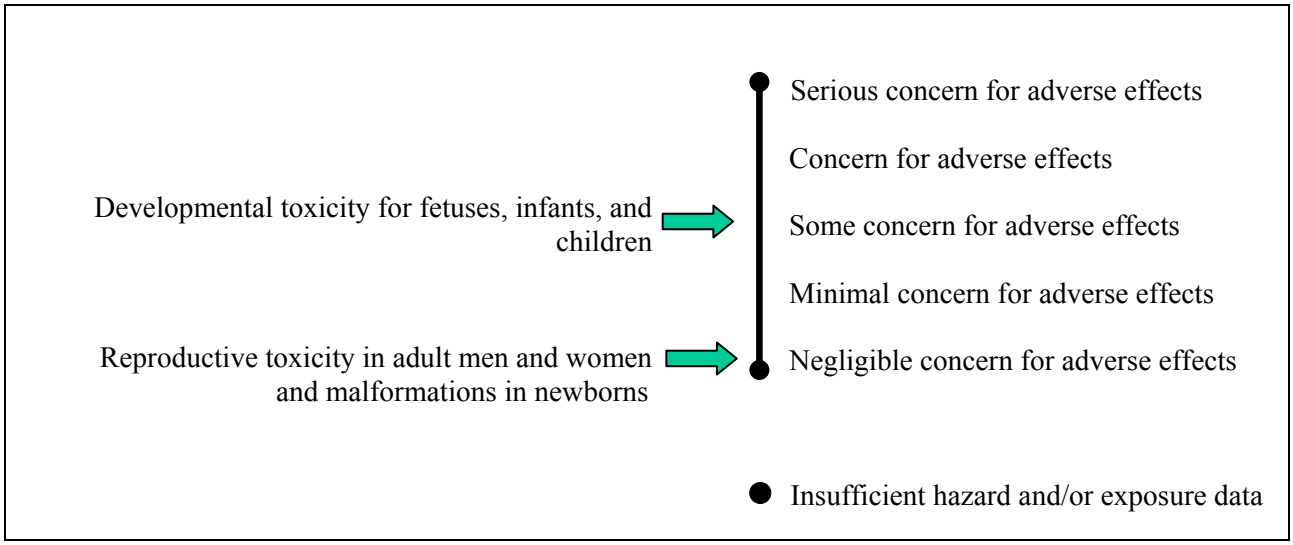


[1]Based on reduced survival in fetuses or newborns (≥ 500 mg/kg bw/day) (*28-32*), reduced fetal or birth weight or growth of offspring early in life (≥ 300 mg/kg bw/day) (*28, 29, 33*), and delayed puberty in female rats (≥ 50 mg/kg bw/day) and male rats and mice (≥ 50 mg/kg bw/day) (*29, 33-35*).

[2]Based on possible decreased fertility in mice (≥ 875 mg/kg bw/day) (*32*); altered estrous cycling in female rats (≥ 600 mg/kg bw/day) (*102*), and cellular effects on the testis of male rats (235 mg/kg bw/day) (*103*).

[3]Based a variety of effects related to neural and behavior alterations (≥10 μg/kg bw/day) (*36-42*), precancerous lesions in the prostate (10 μg/kg bw/day) (*43*) and mammary glands (0.0025 – 1 mg/kg bw/day) (*44, 45*); altered prostate gland and urinary tract development (10 μg/kg bw/day) (*46*), and early onset of puberty (2.4 and 200 μg/kg bw/day) (*40, 47*).

Figure 3. NTP conclusions regarding the possibilities that human development or reproduction might be adversely affected by exposure to bisphenol A



Appendix 1: Interpretation of Blood Biomonitoring Studies

Free bisphenol A has been measured in the blood of pregnant women at concentrations up to 22.4 μg/L (*10*). How to account for the detection of free bisphenol A in human blood is an area of scientific debate. In a controlled and intentional dosing study in humans, free bisphenol A was not detected in the blood or urine of a small number of adult subjects (n=9) orally dosed with 5 mg/person bisphenol A, ~54 – 90 μg/kg (*240*). This dose range is approximately 200 to 400-fold higher than the estimates of daily intake based on urinary biomonitoring data for adults (95th percentile of 0.233 – 0.289 μg/kg bw/day) (*27*). The findings by Völkel *et al.* (*240*) lead to the prediction that the capacity for conjugation reactions is so large in humans that free bisphenol A should not be present in detectable concentrations in the blood of non-occupationally exposed adults. However, biomonitoring studies of the general population report detecting free bisphenol A in the blood, including from pregnant women (*10, 239*), urine (*241*), and breast milk (*5*). Despite the relatively high limit of detection of the analysis method for free bisphenol A of 2.28 μg/L (10 nM) for blood in the 2002 study by Völkel *et al.* (*240*), it is a source of scientific uncertainty why free bisphenol A was not detected in this study in light of reports of mean blood concentrations of free bisphenol A up to 4.4 μg/L (*239*) and 5.9 μg/L (*10*) in pregnant women in the general population.

This discrepancy has contributed to the concern expressed by some scientists that the reported detections of free bisphenol A are artifacts of problems related to sample preparation or storage and the analytical technique employed (*2, 11*). Ideally, methods should measure only bisphenol A and not other compounds ("specificity"). There is scientific consensus that measurements of bisphenol A based on the enzyme-linked immunosorbent assay (ELISA) are the least reliable and non-specific due to potential cross-reactivity with structurally-similar compounds (*2, 3, 11*)[19]. Analytical methods should also be able to detect bisphenol A at low concentrations ("sensitivity"). In addition, measurements of free bisphenol A should be based on analytical methods that accurately distinguish between the concentrations of free bisphenol A and its conjugated metabolites.

There is concern that current measurements of free bisphenol A may be too high (*2, 11*). This could occur, for example, if the method used misidentified other chemicals as bisphenol A or if there was background contamination from laboratory ware. Alternatively, the procedures used to process the samples could introduce bias in measurement even if the analytical method employed is high quality. Measurements of free bisphenol A could be overestimated if the samples were processed in a manner that allowed the conjugated metabolites to revert back to the free form of bisphenol A. For example, conjugated bisphenol A in urine only appears to be stable when stored at room temperature for ~24 hours. After 2 – 4 days at this temperature conjugated bisphenol A begins to degrade and the percent detected in samples decreases ~ 8 to 30%, i.e., higher concentrations of free bisphenol A would be detected over time (*242*).

However, free bisphenol A has been detected in 10% of human urine samples [range = < limit of detection (0.3) – 0.6 μg/L; n = 30] (*241*) and in 60% of breast milk samples [mean = 1.3 μg/L;

[19] Analytical techniques used to measure bisphenol A include gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS), high performance liquid chromatography (HPLC) with fluorescence or electrochemical detection, and enzyme-linked immunosorbent assay (ELISA).

median = 0.4 μg/L; range = < limit of detection (0.3) – 6.3 μg/L; n = 20] (*5*) by researchers at the CDC who use analytical methods considered by many scientists to be very accurate (the CDC has not presented data on measurements of bisphenol A in blood). A proposed explanation to account for the detection of free bisphenol A in breast milk is that the free form of bisphenol A is more lipophilic than the conjugated forms and therefore more likely to sequester in breast milk (*5*).

In addition, Tsukioka *et al.* (*237*) were able to detect free bisphenol A in the urine of all human subjects treated with ~ 0.83 μg/kg, whereas Völkel *et al.* (*240*) was unable to detect any free bisphenol A in subjects treated with doses 65 – 108-times higher, ~54 – 90 μg/kg. It cannot be definitively determined if the detection of free bisphenol A in urine in the study by Tsukioka *et al.* (*237*) was due to the analytical method employed or partial cleavage of glucuronide during sample storage, preparation or analysis. However, Tsukioka *et al.* (*237*) also detected total and free bisphenol A in the urine of subjects that were not intentionally treated [total bisphenol A: 0.82 μg/L (range 0.14 - 5.47; n = 91); free bisphenol A: 0.08 μg/L (range 0.01 - 0.27 ng/m; n = 11)] and these values are lower than CDC measurements of total [2.6 μg/L for all subjects in the NHANES study (*6*)] and free bisphenol A [10 of 30 subjects at <LOD(0.3) - 0.6 μg/L (*241*).

In summary, the NTP recognizes the possibility that the published values of free bisphenol A may, in some cases, not accurately represent the "true" concentrations of free bisphenol A in the blood or body fluids of humans or laboratory animals. However, because of the similarity among values reported with different analytical methods, with the exception of ELISA-based studies, the NTP accepts the published values as sufficiently reliable for use in this evaluation.

Comparison of measured human blood concentrations of free bisphenol A with estimated concentrations in laboratory rodents at low doses

More than ten toxicokinetic and metabolism studies have detected quantifiable levels of free bisphenol A in the blood of adult rodents, mostly rats, following oral administration of doses that are considered high when compared to estimated human daily intakes (500 – 1,000,000 μg/kg for rodents versus < 14.7 μg/kg bw/day for humans) (*3, 19, 27*) (Table 1 and Table 2). These studies were used by Vandenberg *et al.* (*3*) to estimate circulating blood levels of free bisphenol A in laboratory rodents at a lower oral dose of 50 μg/kg bw/day based on the assumption of linear proportionality between administered dose and circulating concentration of free bisphenol A. The estimated peak blood levels of free bisphenol A achieved in the first 30 minutes after dosing ranged from 0.01 to 1.14 μg/L (*3*).

Using the estimates provided by Vandenberg *et al*.(*3*) for peak blood levels of free bisphenol A at 50 μg/kg and again relying on the assumption of linear proportionality, the NTP estimated the range of peak concentrations of free bisphenol A at 10 μg/kg, a dose where a number of "low" dose effects are reported, to be five times lower, i.e., 0.002 to 0.228 μg/L. These values are 2950 to 25.9 times lower than the mean blood concentration of free bisphenol A detected in pregnant women in Michigan (5.9 ± 0.94 μg/L; range 0.5 to 22.4) (*10*).

The appropriateness of extrapolating from higher dose studies to predict blood levels of free bisphenol A at lower dose levels rests on the validity of the assumption of proportionality. This assumption is warranted if, for example, blood levels of free bisphenol A are approximately 10 times lower following dosing with 10 mg/kg than after dosing with 100 mg/kg. Three studies are

available that used non-ELISA methods to measure concentrations of free bisphenol A following oral dosing with 10 and 100 mg/kg bisphenol A in adult rats (*59, 243, 244*). In these studies, the peak, or C_{max}, blood concentrations of free bisphenol A were 4.8-times (*243*), 22.7-times (*244*), and 57-times (*59*) lower in rats treated with a 10 mg/kg dose compared to rats treated with 100 mg/kg.

Directly evaluating proportionality at lower oral doses (< 10 mg/kg) has not been possible in adult animals because blood concentrations of free bisphenol A are below the limits of detection for the analytical methods employed. One strategy that can be used to address the assumption of proportionality at low doses is to rely on studies that have dosed young rodents because they have higher peak blood concentrations of free bisphenol A compared to adults treated with the same dose (*12*). Two studies have measured concentrations of free bisphenol A in young rodents at more than one dose level (*12, 58*). In 3-day old female mice orally treated with 0.035 and 0.395 mg/kg bisphenol A, Taylor *et al.* (*58*) found that the peak blood concentration of free bisphenol A at 0.035 mg/kg was 8.3-times lower than the peak concentration at 0.395 mg/kg (difference between administered does is 11.3-times). The study by Domoradzki *et al.* (*12*) treated neonatal rats orally with higher doses of bisphenol A than those used by Taylor *et al.* (*58*). In 4-day old female and male rats, the peak concentrations of free bisphenol A were 170 to 1610-times lower at 1 mg/kg compared to 10 mg/kg bisphenol A. This finding, coupled with data for 21-day old rats presented in Domoradzki *et al.* (*12*) and the comparisons presented above from Tominaga *et al.* (*244*), and Pottenger *et al.* (*59*), suggest that rodents, and presumably humans, can more efficiently metabolize lower doses of bisphenol A compared to high doses. These data also suggest that extrapolating from higher dose levels in the mg/kg range may overestimate the circulating concentrations of free bisphenol A following administration of oral doses in the low μg/kg range.

Any extrapolation and use of assumptions involves some degree of uncertainty. However, the conclusion outlined above of similar blood levels in the general population and in laboratory animals at "low" doses would still hold even if the estimated blood levels of free bisphenol A in laboratory rodents were overestimated by a factor of 100 or 1000, i.e., the "real" peak blood values in laboratory animals range from 0.2 to 22.8 or 2 to 228 μg/L instead of the estimated 0.002 to 0.228 μg/L.

This possibility that blood concentrations of free bisphenol A in humans could be significantly higher, as much as ~3000 times greater, than the estimated peak concentrations in laboratory animals where biological changes are observed is a point of intense scientific controversy. In brief, although the theoretical plausibility of receptor-mediated effects at "low" doses has been described (*245, 246*), many scientists expect that a compound with a significant degree of biological "activity" at low doses would show more profound impacts on overall toxicity at lower doses than that observed for bisphenol A. With bisphenol A, "low" dose developmental effects can be observed at 0.0024 to 0.010 mg/kg bw/day but indications of severe developmental toxicity in rats and mice, i.e., fetal or neonatal death are not observed except when doses are used that are 50,000 – 200,000-times higher at ≥ 500 mg/kg bw/day (*28-32*).

Estimated daily intake based on back calculating from blood and urine

Based on parameters derived from laboratory animal studies, estimated daily intakes based on back calculations from free bisphenol A measured in human blood are much greater (~500 μg/kg –

1.54 mg/kg bw/day for a 65 kg human) (*3, 236*) than estimates based on any other approach. In contrast, there is a degree of concordance in estimates of daily intake based on other approaches. For these reasons, the NTP has less confidence in daily intake estimates based on blood biomonitoring data compared to other estimates, particularly those based on urine biomonitoring data.

Estimates of daily BPA intake in adults based on aggregating routes of exposure fall within the range of 0.008 – 1.5 μg/kg bw/day (*17, 23*) (Table 1) with most estimates falling within a range that spans one order of magnitude, 0.183 – 1.5 μg/kg bw/day (*16-19, 22*). Daily intakes estimated from the CDC NHANES biomonitoring data are similar and range from 0.289 – 0.233 μg/kg bw/day for adults aged 20 – 60+ years at the 95th percentile (*27*). The NTP considered the possibility that the assumptions used to derive these intakes could underestimate human exposures. For estimates based on aggregating sources of exposure, one concern is that too much emphasis has been placed on diet as the predominant route of exposure. For estimates based on the total concentration of bisphenol A in urine, it is assumed that the daily excretion of bisphenol A is a reasonable surrogate for daily intake. Deviations from the assumptions used to derive current estimates could increase the daily intake estimates, but still result in estimated intakes in the very low μg/kg bw/day range rather than near 1 mg/kg bw/day as predicted from the blood biomonitoring data in adult humans.

Data from an intentional dosing study conducted by Tsukioka *et al.* (*237*) provides further support for daily intakes of < 1 μg/kg. Tsukioka *et al*. gave 15 volunteers (12 men and 13 women) 50 μg of bisphenol A by mouth (~ 0.83 μg/kg for a 65 kg person) and collected urine samples for 5 hours. The average concentration of total bisphenol A was 57.2 μg/L (range 26.5 - 80 μg/L) and free bisphenol A was 1.13 μg/L (range 0.13 - 5.8 μg/L). The administered dose, ~0.83 μg/kg, and urinary concentration of total bisphenol A, 57.2 μg/L, are ~14.8-times and 18.5-times higher, than the estimated median intake of 0.056 μg/kg bw/day for adults aged 20-39 years based on a median urinary concentration of 3.1 μg/L calculated by Lakind *et al.* (*27*). Extrapolating downward for administered dose and urinary concentrations of total bisphenol A from the data provided by Tsukioka *et al.* (*237*) would give values that are consistent with the daily intake calculated by Lakind *et al.* (*27*) based on the CDC urinary measurements (*6*).

Exposure Assessment Research Needs

The NTP concurs with the CERHR Expert Panel on Bisphenol A that more measurements in humans are needed of free and total bisphenol A, its glucuronide conjugate, and other metabolite concentrations from maternal, fetal, and neonatal tissues or fluids (i.e., placenta, amniotic fluid, breast milk, urine, serum). These data would provide further insight into the roles of metabolism and exposure route on internal dose and provide a firmer foundation for extrapolations of risks to humans from the wealth of animal studies available. Available data demonstrate that a large fraction of children and adults have detectable levels of bisphenol A, or it's metabolites, in their urine. Duplicate diet studies to identify in detail the sources and routes of exposure of bisphenol A would be useful. For example, while research suggests diet is the major source of bisphenol A for infants and young children in the United States, the detailed analysis of bisphenol A levels has primarily focused on polycarbonate baby bottle leachates and canned food. The contributions of non-canned food and drinking water routes of exposure for youth and adults not occupationally-exposed to BPA remain unknown and in need of further study. Levels of bisphenol A in

residential drinking water wells and community water sources have not been systematically studied. Also unknown is the impact of landfill leachates on levels of bisphenol A in U.S. drinking well waters and whether chlorinated congeners of bisphenol A are found in municipal water supplies.

More research is needed to characterize the toxicokinetics of bisphenol A in developing animals under exposure scenarios that better mimic the low-level chronic exposures experienced by humans. Currently, only single or "acute" dosing kinetic studies in laboratory animals are available for predicting the metabolism and fate of bisphenol A following long-term, daily exposure, or for comparing apparent differences in the metabolism and fate of bisphenol A in laboratory rodents and humans. Repeated administration of many compounds has been shown to alter the capacity of the animal to metabolize and excrete the compound. Further characterization of the ability of repeated exposures to bisphenol A to change rates and extents of metabolism and excretion in laboratory animals and humans is a critical research need.

In addition, it is clear that there are differences in the pharmacokinetics of bisphenol A, particularly between rats and humans that complicate using the rat data to interpret the human biomonitoring data. For example, the excretion profiles of bisphenol A differ in rodents and humans. In humans, the major route of elimination is via the urine in the form of bisphenol A glucuronide (*247*). In contrast, the major elimination routes in rodents are as bisphenol A in the feces and as bisphenol A glucuronide in the bile and, to a lesser extent, in the urine [reviewed in (*2*)]. Also, in rats bisphenol A glucuronide can remain in the bile and be re-circulated back to the liver ("enterohepatic circulation"). Development of physiologically-based pharmacokinetic (PBPK) models is needed to facilitate the interpretation and applicability of animal studies for human risk assessment.

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