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Proceedings

Bisphenol A: Low Dose Effects—High Dose Effects

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Local Organizing Committee:

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Environmental endocrine active compounds (EAC) have the potential to interfere with the endocrine, immune, and nervous systems. It is theorized that these agents are responsible for reproductive abnormalities in both humans and wildlife and the increase in the incidence of breast and testicular cancers. There is concern that current risk assessment strategies for compounds released into the environment are not adequate to evaluate possible hormonal disruption capabilities. Two critical issues concerning endocrine disruption are the existence of possible low dose effects that do not occur at high doses and the impact on the developing organism. Bisphenol A (BPA), an estrogenic chemical used in the manufacture of epoxy resins and polycarbonates, is a substance ubiquitously found in consumer products and was the sole endocrine active compound discussed at this workshop. Studies investigating its potential adverse effects have yielded conflicting results. Alterations in the reproductive system have been observed in some investigations on BPA exposure at doses far below the current accepted NOAEL of 50 mg/kg while no effect has been seen in other studies. The aim of this workshop was to clarify which factors may be responsible for these conflicting results and to identify and prioritize future research strategies. This workshop did not serve as a regulatory risk assessment evaluation.

Program

SATURDAY, NOVEMBER 18, 2000

Chair: Ibrahim Chahoud, Freie Universität Berlin, Germany

OPENING

10:00 *Martin Paul*
Dean of Benjamin Franklin Medical Center, Freie Universität Berlin, Germany

10:15 *Dietrich Rosenkranz.*
Chairman of the Department of Ecology
Federal Environmental Agency, Germany

10:30 *Ellen Silbergeld, University of Maryland, USA*
Endocrine Disruption: current strategies in the US

11:00 *Ursula Gundert-Remy, Federal Health Agency for Consumer Protection and Veterinary Medicine, Germany*
Endocrine Disruption—a challenge for toxicology

11:30 Coffee Break

11:45 Session I ECOTOXICOLOGY
Chair: *Andreas Gies, Federal Environmental Agency, Germany*

11:55 *John Sumpter, Brunel University, UK*
A multi-generation study of the effects of Bisphenol-A on the fathead minnow (*Pimephales promelas*)

12:25 *Werner Kloas, Institute of Freshwater Ecology and Inland Fisheries, Germany*
In vitro and *in vivo* effects of bisphenol A on the amphibia Xenopus laevis

12:55 Lunch

14:00 *Dan Pickford, Astra Zeneca Brixham Laboratories, UK*
Effects of bisphenol A on larval growth, development and sexual differentiation of the African clawed frog (*Xenopuslaevis*)

14:30 *Jörg Oehlmann, Zittau University, Germany*
Effects of low concentrations of bisphenol A in prosobranch molluscs

15:00 General Discussion

15:30 Session IIa EXPOSURE AND TOXICOKINETICS
Chair: *John Ashby, Astra-Zeneca, UK*

15:40 *Nicolas Olea, University of Granada, Spain*
Human exposure to bisphenols

16:10 *Andrea Wenzel, Fraunhofer Institute IUCT, Germany*
Bisphenol A concentrations in the aquatic environment

16:40 *John M. Waechter, BPA Global Industry Group*
Route-dependency in the bioavailability of bisphenol A in Fischer 344 rats and differential metabolism of BPA in primary cultured hepatocytes from rats, mice and humans

17:10 General Discussion

SUNDAY, NOVEMBER 19, 2000

9:00 Session IIb EXPOSURE, TOXICOKINETICS AND UTEROTROPHIC ASSAY
Chair: *Gisela Degen, University of Dortmund, Germany*

9:00 *Wade V. Welshons, University of Missouri-Columbia, USA*
Low dose effects of environmental estrogens: Why bisphenol A is weak in the adult yet strong in the fetus

9:30 *Gilbert Schönfelder, Freie Universität Berlin, Germany*
Bisphenol A concentration in human umbilical cord blood and placenta

9:50 *Gisela Degen, University of Dortmund, Germany*
Comparative toxicokinetics of bisphenol A in pregnant and non-pregnant DA/Han rats

10:10 Coffee Break

10:30 *Wolfgang Dekant, University of Würzburg, Germany*
Comparative toxicokinetics of bisphenol A in humans and rats

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10:50 *Horst Michna, DSHS-Cologne, Germany*
The effects of low and high doses of BPA on (anti-) androgenic and (anti-) estrogenic parameters in the reproductive tract of mice (NMRI) and rats (Wistar, SD, Da-Han)
11:10 *Ken Brown, George Washington University, USA*
Effects of bisphenol A on uterine weight, morphology and heat shock protein levels
11:30 General Discussion
12:00 Lunch
13:00 Session IIIa PRE- AND PERINATAL EFFECTS OF BISPHENOL A
Chair: *Fred vom Saal, University of Missouri-Columbia, USA*
13:10 *John Ashby, Astra-Zeneca, UK*
Possible influences leading to divergent findings in low dose endocrine disruption studies
13:55 *Fred vom Saal, University of Missouri-Columbia, USA*
High sensitivity of the fetal prostate to endogenous and environmental estrogens
14:40 *Rochelle Tyl, Research Triangle Institute, USA*
Three-generation reproductive toxicity evaluation of bisphenol A administered in the feed to CD (Sprague Dawley) rats
15:10 *Chris E. Talsness, Freie Universität Berlin, Germany*
The effects of low and high dose in utero exposure to bisphenol A on the reproductive system of female Sprague Dawley rat offspring
15:30 *Ibrahim Chahoud, Freie Universität Berlin, Germany*
The effects of low and high dose in utero exposure to bisphenol A on the reproductive system of male Sprague Dawley rat offspring
16:00 General Discussion
16:30 Poster Session
20:00 Dinner

MONDAY, NOVEMBER 20, 2000

9:30 Session IIIb PRE- AND PERINATAL EFFECTS OF BISPHENOL A
Chair: *Martin Paul, Freie Universität Berlin, Germany*
9:40 *Gilbert Schönfelder, Freie Universität Berlin, Germany*
Molecular aspects of low and high dose bisphenol A prenatal exposure
10:10 *John M. Butala, BPA Global Industry Group*
Normal reproductive organ development in CF-1 mice following prenatal exposure to bisphenol A and in Wistar rats exposed to bisphenol A in the drinking water
10:40 *Ana Soto, Tufts University, USA*
Proliferative effects of *in utero* exposure to Bisphenol A on mammary gland development
11:10 *Frank Welsch, Chemical Industry Institute of Toxicology, USA*
Lack of effects of perinatal exposure to low doses of bisphenol A on male rat offspring ventral prostate
11:40 General Discussion
12:10 Lunch
13:10–15:30 Roundtable Discussion
Resolution of conflicting data and necessary future research
directions
Chair: *Ellen Silbergeld, University of Maryland, USA*

EFFECTS OF BISPHENOL A ON UTERINE WEIGHT, MORPHOLOGY AND HEAT SHOCK PROTEIN LEVELS

K.M. Brown[a], A.D. Papaconstantinou[a], B.R. Fisher[b], P.L. Goering[c], T.H. Umbreit[c]. [a] Dept. Biological Sciences, George Washington Univ., Washington, D.C., USA; [b]Covance Laboratories, Inc., Vienna, VA, USA; [c]Health Sciences Branch, CDRH, FDA, Rockville, MD, USA

We have compared the effects of the xenoestrogen, bisphenol A (BPA), and β-estradiol (E_2) on uterine wet weight, morphology and heat shock protein levels in ovariectomized B6C3F1 mice. To examine whether these effects were mediated through the estrogen receptor (ER), the ER antagonist, ICI 182,780 (ICI), was co-administered with BPA or E_2. Mice were injected subcutaneously daily for 4 days with corn oil (control), E_2, or BPA, alone or in combination with ICI. Uterine morphometric analysis and hsp90α immunocytochemistry were performed on paraffin sections, and levels of hsp90α, hsp72, hsc73 and grp94 were determined by Western blot analysis. BPA increased uterine weights at doses between 40 and 400 mg/kg/day with an EC_{50} of 36 mg/kg/day compared to 0.97 μg/kg/day for E_2, although the maximum response to BPA was approximately one-third that to E_2. Both chemicals also increased uterine epithelial height, stromal and myometrial thickness, and levels of hsp90α, hsp72 and grp94. E_2 and BPA treatments resulted in a decrease in hsp90α immunostaining in nuclei of epithelial cells and an increase in the cytoplasm and nuclei of cells of the stroma and myometrium. All effects of E_2 and all BPA effects, except on stroma and myometrium thickness, were lowered by ICI. These results suggest that the BPA-induced increases in uterine weight, epithelial cell height, and heat shock protein levels are mediated by the estrogen receptor.

NORMAL REPRODUCTIVE ORGAN DEVELOPMENT IN CF-1 MICE FOLLOWING PRENATAL EXPOSURE TO BISPHENOL A AND IN WISTAR RATS EXPOSED TO BISPHENOL A IN THE DRINKING WATER

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Sex organ weight, daily sperm production (DSP), epididymal sperm count and testis histopathology were assessed in 90-day old male offspring of female CF-1 mice exposed to Bisphenol A (BPA) (0, 0.2, 2, 20 or 200 micrograms/kg/day) on gestation days 11–17. An additional group received 0.2 micrograms/kg diethylstilbestrol (DES) orally. Clinical observations, body weight and food consumption in pregnant dams were unaffected by BPA or DES. There were no BPA or DES-related effects on growth or survival of offspring. The total number of pups per litter was slightly below controls in the 200 microgram/kg/day BPA group. This was not considered treatment-related since the litter size was within the normal range of historical controls. There were no treatment effects of BPA or DES on testes histopathology; DSP or sperm count; or on prostate, preputial gland, seminal vesicle or epididymis weight at doses previously reported to affect these organs or at doses an order of magnitude higher or lower.

10 week old Female Han-Wistar rats received drinking water containing 0, 0.01, 0.1, 1.0, or 10 ppm BPA or 0.1 ppm DES 7

days/week for 10 weeks. Exposures commenced 2 weeks prior to mating and continued through lactation. Offspring males were raised to 90 days of age with untreated drinking water and evaluated. There were no growth or reproductive effects of BPA treatment on parental females. Growth; survival; DSP; sperm count; and weight of testes, preputial gland, and prostate were unaffected in pups from BPA-treated dams. DES administration caused reduction in body weight and food consumption in dams, an increase in gestation period and a decrease in live pup number. The conclusions from both studies were that BPA did not cause effects on male reproductive organs or reproductive parameters at any dose tested, whereas DES at an effective dose caused reduced body weight, lengthened gestation and decreased live pup numbers in rats.

THE EFFECTS OF LOW AND HIGH DOSE *IN UTERO* EXPOSURE TO BISPHENOL A ON THE REPRODUCTIVE SYSTEM OF MALE RAT OFFSPRING

Ibrahim Chahoud, Olaf Fialkowski, Chris E. Talsness. Institute of Clinical Pharmacology and Toxicology, Benjamin Franklin Medical Center, Freie Universitaet, Berlin, Germany

We examined the effects of prenatal exposure to BPA (0.02, 0.1 and 50 mg/kg/d) or ethinyl estradiol (EE) (0.02 and 0.2 mg/kg/d) per gavage on gestation days 6 through 21 on the reproductive system of male Sprague Dawley rat offspring. The control gravid dams received a 2% cornstarch suspension (1.0 ml/100 g BW). Reproductive developmental landmarks including anogenital distance on PND's 3, 15 and 21, testicular descent and preputial separation were recorded. The male offspring were sacrificed either on PND 70 or 170 to examine reproductive organ weights, spermatid and sperm numbers, daily sperm production, sperm morphology and testosterone concentrations. In general, feminization of the perineum was observed following exposure to all doses of BPA and to the high dose of EE, while the low dose of EE resulted in longer anogenital distances. Descent of the testes was delayed in all treatment groups except for the high dose (50 mg/kg) BPA. Preputial separation was delayed in the low dose groups (0.1 and 0.02 BPA and 0.02 EE), while it occurred earlier in the 0.2 EE group. Juvenile absolute testicular weight was significantly decreased in the lowest dose groups (0.02 BPA and 0.02 EE) and this effect remained to adulthood. Juvenile absolute epididymal weight was significantly increased in the 50 BPA group and decreased in the 0.1 and 0.02 BPA groups and the low dose EE (0.02 EE). At adulthood, the decrease in the low dose BPA (0.02) group remained. Juvenile absolute prostate weight was increased in the 50 BPA group while it was decreased in the low dose (0.02) BPA and EE groups. This effect for BPA on the prostate was no longer observed in adulthood, while it remained for the 0.02 EE groups and appeared in the 0.2 mg/kg EE group. Juvenile absolute seminal vesicle weight was decreased in the low dose (0.02) BPA and EE groups with the effect remaining only in the BPA group during adulthood. In adulthood, daily sperm production was significantly decreased in all treatment groups except at the NOEL dose (50) for BPA, while testosterone levels were increased in all treatment groups. Prenatal exposure to BPA at doses lower than the currently accepted NOEL alter reproductive system function well into adulthood. The appropriateness of existing guidelines to identify acceptable levels of exposure needs to be reexamined for substances with endocrine activity. This work was supported by a grant from the BMBF (07HORO1/7).

COMPARATIVE TOXICOKINETICS OF BISPHENOL A IN PREGNANT AND NONPREGNANT DA/HAN RATS

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Despite the low oestrogenic potency of bisphenol A (BPA) there is concern that this compound, as a consequence of a potentially low clearance, might reach biologically significant levels in human and animal tissues upon environmental exposures. To address this concern we have assessed the toxicokinetics of BPA in female non-pregnant DA/Han rats upon *i.v.* and gavage administration. We also studied, by GC-MS analysis, the disposition of BPA in pregnant DA/Han rats and its placental transfer to fetuses in late (day 18) gestation.—The toxicokinetic properties of BPA in non-pregnant rats were found in agreement with the hypothesis of a rapid hepatic first pass elimination and with the efficient metabolic clearance of low oral doses (10 mg/kg b.w.; Upmeier et al., Arch Toxicol, online publishing dated 18.08.2000). Fluctuations in BPA plasma concentrations over time pointed to enterohepatic recirculation, and protracted absorption from the gastrointestinal tract was noticed at the higher dose of 100 mg/kg b.w.—The experiments in pregnant rats used a "destructive design" to allow a comparison of BPA levels in maternal tissues and fetuses between 5 and 120 min after *i.v.* administration of a 10 mg/kg b.w. dose. The data were indicative of a rapid transfer from the mother to the fetus and of an efficient extraction of BPA from the blood by the maternal liver. Maximal BPA concentrations found in fetal liver were far below those in the maternal compartment, and the general patterns of BPA concentration-time-curves resembled those found for the phytoestrogen daidzein in pregnant DA/Han rats.
Supported by Umweltbundesamt and the Verband der Chemischen Industrie

COMPARATIVE TOXICOKINETICS OF BISPHENOL A IN HUMANS AND RATS

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Bisphenol A is widely used with many potential sources of human exposures. To contribute to the characterisation of potential adverse effects of bisphenol A on humans, its disposition and excretion was compared in humans and rats. To study kinetics of excretion, bisphenol A (200 mg/kg in corn oil) was administered to three male and three female rats and excreted urine was collected for 96 h. Unchanged bisphenol A and formed metabolites (bisphenol A glucuronide and sulfate) were quantified by HPLC. In urine, app. 30 % of the administered dose of bisphenol A (mainly in the form of conjugates) were recovered. Bisphenol A and its metabolites were slowly excreted in rats in several phases suggesting enterohepatic circulation. To study human disposition and kinetics of bisphenol A excretion, human volunteers (three males and three females) were administered d_{16}-bisphenol A (5 mg). Blood and urine samples were taken in intervals (up to 96 h) and d_{16}-bisphenol A content was determined by GC/MS with chemical

ionisation and negative ion detection. d_{16}-Bisphenol A was rapidly cleared from human blood and urine with a half-life of 3.5 hours; app. 25% of the applied d_{16}-bisphenol A dose was recovered in the urine of the volunteers. The obtained data indicate limited absorption of bisphenol A from the gastrointestinal tract of rodents and humans after oral administration and a very efficient excretion of bisphenol A by humans, likely due to the absence of enterohepatic circulation of bisphenol A metabolites.

This work was supported by the German Umweltbundesamt.

COMPARISON OF THE HORMONAL SUSCEPTIBILITY OF DIFFERENT RAT STRAINS TO BISPHENOL A TREATMENT

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Recent data favor the idea, that different strains of the rat may be differently sensitive to estrogenic stimuli. For this reason we have compared the estrogenic potency of the phytoestrogen genistein (GEN) and the xenoestrogens bisphenol A (BPA) and p-tert-octylphenol (OCT) in Da/Han (DH), Sprague Dawley (SD) and Wistar (WIS) rats. Rats were treated p.o. for three days with different doses of the respective compounds and ethinylestradiol (EE). Uterus wet weight, the thickness of the uterine epithelium, the uterine gene expression of Clusterine (CLU) and complement C3 (C3) and the thickness of the vaginal epithelium were examined as parameters for the estrogenic potency. After treatment with BPA, OCT and GEN a comparable response of all analysed parameters could be observed in DH, WIS and SD rats. The uterus wet weight of SD rats responds with a reduced sensitivity to EE treatment in comparison to DH and WIS rats. In conclusion, our results indicate that all analysed rat strains respond with a comparable sensitivity to phyto- and xenoestrogen treatment. Summarising our results WS rats seem to be the most useful and sensitive rat strain to analyse the estrogenic potency of substances. This study was part of the research project: “Vergleich der östrogenen Potenz ausgewählter synthetischer Chemikalien und natürlich vorkommender Östrogene: Bewertung einzelner Verbindungen” founded by the: Verband der Chemischen Industrie, Germany

ENDOCRINE DISRUPTION—A CHALLENGE FOR TOXICOLOGY

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Risk assessment of xenobiotics is a qualitative and quantitative assessment of toxic properties conventionally based on data resulting from tests in animals exposed to the substance. The assessment of dose-effect relationship includes evaluation of exposure at the site of action. More recently, emphasis is put on understanding the relationship between exposure at the site of action and the resulting effect, i.e. toxicodynamic. In this respect, toxicodynamic endpoints such as binding to receptors have been used as indicating toxic effects (surrogate endpoints).

In recent years, public health discussion has focussed on chemicals in the environment, which are capable to bind to oestrogen receptors. This binding property has been used as a surrogate for a pharmacological action. As a corollary, ‘environmental estrogens’ have been thought to have the same action known from endogenous estrogens and thereby interfere with normal function leading to reduced fertility and changes in sexual differentiation and maturation (Colborn et al., 1993).

We screened the literature on bisphenol A (BPA) for toxicodynamic endpoints, such as receptor binding, data on concentration/dose and on physiological responses. Finally, qualitative and/or quantitative data on “classical” endpoints were selected from the literature. It was the aim to demonstrate the quantitative link of toxicodynamic data in vitro with toxicodynamic data in vivo and to compare the results with the findings from conventional endpoints after testing for influences on fertility and reproduction, including sexual development.

Bisphenol A has been detected as binding to oestrogen receptors (Krishnan et al., 1993) and having estrogenic activity in several in vitro tests (Soto et al., 1995). Hence, it has been qualified as being an endocrine disrupter. However, it is until now difficult to derive a clear-cut relationship between binding to the receptor and endpoints in classical testing. This might be due to the fact that the effect may depend not only on the dose but also on the timing of the dose. Thus, the effect may vary with dose and with time so that a multidimensional approach would be necessary to evaluate the relationship between dose, time and effect.

THE ESTROGENIC ACTIVITY OF BISPHENOL-A *IN VITRO* AND *IN VIVO*

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The potential estrogenic activities of bisphenol A (BPA) were investigated *in vitro* (E-screen and estrogen receptor competitive binding bioassays) and *in vivo* (uterotrophic assay and Hershberger assay). The uterotrophic effect was evaluated using mature ovariectomized (OVX) Sprague-Dawley female rats treated subcutaneously with bisphenol A (1, 5, 10, 50, and 100 mg/kg/day), E2 (0.3□/kg), and DES (0.3□/kg) for 3 consecutive days. The Hershberger assay was performed in immature rats castrated at 6 weeks of age. In MCF-7 cell proliferation assay (assessed by sulforhodamine B assay), E2 and DES used as positive estrogens induced maximum proliferation of MCF-7 cells at 1.0 nM, whereas BPA slightly induced MCF-7 cell proliferation only at concentration of 0.1 μM. In a competitive binding assay, E2 and DES showed inhibition of 17β-[^{3}H]estradiol binding to the rat uterus ER with an IC50 of 1.0 nM and 0.5 nM, respectively. However, BPA had an IC_{50} of 5 μM, which was approximately 5,000–10,000-fold greater than that of E2 and DES. In uterotrophic assay, uterus weights were significantly increased at the dose of BPA 100 mg/kg/day in OVX female rats. However, BPA did not show any antiandrogenic effects (inhibition of sex accessory tissues re-growth induced by testosterone) in Hershberger assay. These studies demonstrate that BPA exhibits weak estrogenic activity *in vitro* and *in vivo* assay systems, but not act as an antiandrogen (supported by EDs research funds of Korea FDA).

Keywords: Bisphenol-A, E-screen, ER binding, Uterotrophic assay, Hershberger assay

BIODEGRADATION OF BISPHENOL A IN AQUATIC ENVIRONMENTS: RIVER DIE-AWAY

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The biodegradability of bisphenol A (BPA) was assessed in surface waters from seven different rivers across the United States and Europe. River locations ranged from light to heavily industrialized, and included estuarine sites. Reaction mixtures were prepared from samples of water and surficial sediments collected upstream and downstream of outfalls from wastewater treatment plants known to receive BPA. Biodegradation of BPA was followed in a respirometer that measured oxygen consumption and CO_2 production. Parallel studies measuring the mineralization of [^{14}C]BPA to $^{14}CO_2$ provided comparable results and confirmed the suitability of the respirometric method. Reaction mixtures were incubated at 20 ± 2°C in the dark and continually mixed to ensure maintenance of aerobic conditions. Rapid biodegradation of BPA was observed in the reaction mixtures following lag phases ranging from 2 to 4 days. Biodegradation half-lives for BPA were typically less than two days following the lag phase. Mineralization of BPA was observed in all river waters with average carbon dioxide yields of approximately 76 percent of the theoretical maximum (range 59–103%) at the end of the incubation period (≤18 days). Minimal degradation of BPA was observed in control mixtures sterilized by autoclaving, confirming that the disappearance of BPA was due to biological processes. Short half-lives (1.2 ± 0.7 days) were noted for BPA degradation in river waters regardless of geographic location, sampling site (i.e. upstream vs. downstream of wastewater outfalls), sediment addition (≤0.05%), and initial test chemical concentration (50 to 5,500 μg/L). Subsequent studies conducted at environmentally relevant concentrations (0.05 and 0.5 μg/L) also indicated short half-lives (3–6 days) for BPA, and support the extrapolation of the half-lives measured in this study over a wide range of environmental concentrations. The fact that BPA was degraded rapidly in surface waters taken from diverse locations in the United States and Europe, as well as in studies recently conducted in Japan, suggests that BPA degrading microorganisms are widely distributed in nature. These observations, combined with the results of previous results from standardized biodegradation tests, as well as wastewater and environmental simulation studies, provide clear evidence that BPA is not persistent in the aquatic environment.

IN VITRO AND *IN VIVO* EFFECTS OF BISPHENOL A ON THE AMPHIBIAN *XENOPUS LAEVIS*

Werner Kloas, Gregor Levy, Christian Bögi, Ilka Lutz. Department of Inland Fisheries, Institute of Freshwater Ecology and Inland Fisheries, Müggelseedamm 310, Germany

Potential interferences of environmental endocrine active compounds (EAC) have been shown in all classes of vertebrates. The aim of our research using the amphibian *Xenopus laevis* as a model is to study wether EAC are able to cause adverse humoral effects on sexual differentiation in amphibians. The assessment of potential endocrine disrupting effects of bisphenol A (BPA) compared to other estrogenic compounds (estradiol (E2) and nonylphenol (NP)) includes several levels: (I) binding of compounds to estrogen receptor (ER), (II) biological activity *in vitro* by assaying induction of estrogenic biomarkers vitellogenin (Vg)- and ER-mRNA in primary cultured hepatocytes, and (III) *in vivo* effects on sexual differentiation and biomarker induction during larval development. Our results indicate that all compounds tested are able to bind to ER with following ranking: E2 > NP = BPA, whereas dose response curves to induce Vg- and ER-mRNA *in vitro* showed a ranking of E2 > NP > BPA. *In vivo* exposure during larval development resulted in increases of female phenotypes by E2 at 10^{-7}M and 10^{-8}M while treatment with NP and BPA was only significant at 10^{-7}M concommittant with an elevation of estrogenic biomarkers, which could be obtained also after short term (2 weeks) exposure of juvenile *Xenopus* at 10^{-7}M BPA. Recently, several investigations demonstrated the existence of nonclassical membrane associated actions of steroids. In order to test the hypothesis wether E2 could act via cell membranes we investigated a possible binding of [^{3}H]-E2 to liver cell membranes of *Xenopus.* The results demonstrate the existence of specific [^{3}H]-E2 membrane binding and competitive displacements revealed a different specificity of membrane [^{3}H]-E binding compared to the classical cytosolic ER. In addition, BPA could also displace [^{3}H]-E binding suggesting that an additional cellular signalling pathway might exist not only for E2 but also for BPA.
Supported by grants BW-PLUS: Oe 98007 and UBA: FKZ 299 65221/22.

IN VIVO EFFECTS OF ENDOCRINE DISRUPTORS ON SEXUAL DIFFERENTIATION AND BIOMARKERS IN THE AMPHIBIAN *XENOPUS LAEVIS*

Gregor Levy, Christian Bögi, Ilka Lutz, Werner Kloas. Department of Inland Fisheries, Institute of Freshwater Ecology and Inland Fisheries, Müggelseedamm 310, Germany

Several chemical compounds present in the environment are known to affect reproductive biology of animals by endocrine disrupting effects. However, most investigations concerning effects of these so called endocrine disruptors are dealing with *in vitro* assays to determine estrogenic activities. The focus of our present paper is to get more information about effects of endocrine disruptors *in vivo* using the amphibian *Xenopus laevis* as a model. The most obvious adverse effect on reproductive biology of amphibians as an endpoint method is a shift of sex ratio caused by natural estrogens or xenoestrogens during larval development leading to feminization. The treatment of *Xenopus* tadpoles by (xeno)estrogen exposition takes about 2 months until metamorphosis is completed. Thus the aim of our experiments is to look for a faster method to detect possible biomarkers indicating adverse effects *in vivo*. The classical exposure experiment with 17β-estradiol and the xenoestrogens, nonylphenol and bisphenol A, was correlated with determinations of possible estrogenic biomarkers using a semiquantitative RT-PCR technique for vitellogenin (Vg)-, estrogen receptor (ER)-, androgen receptor (AR)-, and retinol binding protein (RBP)-mRNA in parallel during complete metamorphosis. No Vg-mRNA could be detected in tadpoles while mainly AR-, and RBP-mRNA showed elevated levels in whole body homogenates at the end of larval development. Short term exposure of juvenile *Xenopus* for 2 weeks resulted in strongly

increased levels of Vg-mRNA but also for RBP-, AR-, and ER-mRNA isolated from liver. Experiments are in progress to detect a sensitive window during larval development as well as for juveniles to determine endocrine disrupting effects by the use of the best estrogenic biomarker, respectively.
Supported by grants BW-PLUS: Oe 98007 and UBA: FKZ 299 65221/22.

DEVELOPMENTAL EXPOSURE TO A RECONSTITUTED PCB-MIXTURE ALTERS SEX-DEPENDENT BEHAVIORS AND STEROID HORMONE LEVELS IN RATS

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Our previous study revealed a reduction of aromatase activity in the brain of newborn male rats exposed to a PCB-mixture which was reconstituted according to the pattern found in human milk. In adult male littermates feminized sweet preference behavior was detected together with reduced serum concentrations of testosterone and testes weights. The reconstituted mixture was more effective than the technical mixture (Aroclor 1254). In the present study the dose-response relationship was examined for the reconstituted mixture. Female rats were fed diets with 0, 5, 20, or 40 mg/kg diet, resulting in an average daily intake of 0, 0.5, 2, or 4 mg/kg body wt. Exposure started 50 days prior to mating and was continued until birth of the offspring. A dose-dependent elevation of sweet preference was found in adult male offspring indicating feminization of this sexually dimorphic behavior. A conditioned place preference test revealed a preference for the testosterone-paired side at the highest exposure condition. Dose-dependent reductions of serum testosterone and estradiol concentrations were detected in weanling female offspring and of testosterone in adult male littermates. Taken together, results indicate long-lasting and dose-dependent changes in sex-specific behaviors and levels of sex steroid hormones in rats after maternal exposure to a PCB-mixture resembling the breast milk pattern.
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THE EFFECTS OF LOW AND HIGH DOSES OF BPA ON (ANTI-)ANDROGENIC AND (ANTI-)ESTROGENIC PARAMETERS IN THE REPRODUCTIVE TRACT OF MICE (NMRI) AND RATS (WISTAR, SPRAGUE DAWLEY, DA/HAN)

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The objective was to investigate the endocrine potential of BPA in classical test systems. Based on our experience in drug discovery we decided to choose female mice for the detection of estrogenic effects; in addition, different rat strains were studied with the goal to elucidate influences leading to divergent findings in endocrine disruption studies. (Anti-)androgenic potential of BPA was tested in orchiectomized Wistar rats.
(Anti-)Androgen Test: in positive control groups testosterone propionate stimulated prostate growth; BPA stimulated prostate growth, but not statistically significant, at the suprapharmacological dose of 500 mg/kg body weight; BPA did not exhibit androgen-like effects at doses of 3, 50, 200 mg/kg after p.o. treatment. No antiandrogenic effect of BPA could be detected for these doses, whereas flutamide was able to antagonize the growth stimulating effect of testosterone propionate.
(Anti-)Estrogen Test: in ovariectomized mice a uterotrophic response was detected in positive control groups, whereas no effect on uterine growth reactions were displayed after treatment with BPA (0,002 mg, 0,2 mg, 20 mg, 50 mg/kg body weight) with the exception that the dose of 500 mg/kg body weight slightly stimulated the growth of the uterus. In Sprague Dawley rats BPA was not found to stimulate uterine wet weight at any dose applied (5 mg, 50 mg, 200 mg/kg), whereas in the Da/Han and Wistar rats a slight stimulation was detected at the highest dose (200 mg), nevertheless there were no dose dependent effects.
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EFFECTS OF LOW CONCENTRATIONS OF BISPHENOL A IN PROSOBRANCH MOLLUSCS

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Endocrine disruption by xenobiotics has been reported so far almost exclusively for vertebrates. Only little is known about comparable effects in invertebrates with the exception of imposex and intersex in prosobranch snails as a tributyltin response. The objective of this study was to develop an organismic invertebrate test system using the freshwater snail *Marisa cornuarietis* to identify endocrine-mimetic test compounds. During the laboratory experiments, the snails were exposed to a number of endocrine active model compounds, including bisphenol A (BPA) at nominal concentration ranges between 1 and 100 μg/L during 5 months using adult specimens and in a complete life-cycle test for 12 months. In these experiments, BPA induced a complex syndrome of alterations in females referred to as the induction of “superfemales” even at the lowest test concentrations. Affected specimens were characterised by the formation of additional female organs, an enlargement of the accessory pallial sex glands, gross malformations of the pallial oviduct section resulting in an increased female mortality, and a massive stimulation of oocyte and spawning mass production. Because statistically significant effects were observed at the lowest nominal test concentrations (1 μg BPA/L), it can be assumed that even lower concentrations may have a negative impact on the snails. The results show that prosobranchs are sensitive to BPA-induced endocrine disruption at environmentally relevant concentrations and that especially *M. cornuarietis* is a promising candidate for a future organismic invertebrate model to identify endocrine-mimetic test compounds.
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HUMAN EXPOSURE TO BISPHENOLS

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Bisphenols are a group of endocrine disrupting compounds that were initially designed as synthetic estrogenic hormones and now form a part of innumerable manufactured products. Bisphenol-A is the best-studied substance of the whole series of bisphenols. It is used for the production of polycarbonates and polyester epoxy resins, which account for nearly 90% of the European production of bisphenol-A. It is also employed in the manufacture of thermal-pressure sensitive paper, PVC, polyols and polyurethane, brake fluid and tyres and in polyamide processing. The EU consumption of bisphenol-A accounts for more than 600,000 tonnes per year. The estrogenicity of bisphenols was first documented in 1936, when bisphenol-F was already used as base monomer in bakelite, developed in 1909. Exposure among both professional and general populations has been documented. Among the former, dermal and inhalation are the most common route, but for consumers, exposure comes from the ingestion of food and beverages in contact with epoxy resin coatings, polycarbonate containers, tableware devices, baby bottles, pipe lining and from medical applications, such as the use of bisphenol-A in dental composites and sealants. The bioaccumulation of bisphenols has also been documented, specially for those containing chlorine and bromine, as a result of their fat solubility. It can be affirmed that: i) bisphenols is a broad term that includes various compounds that are structurally similar to bisphenol-A; ii) human exposure to bisphenols is a significant and demonstrated phenomenon; iii) the estrogenicity of bisphenols is of particular concern.

EFFECTS OF BISPHENOL A ON LARVAL GROWTH, DEVELOPMENT AND SEXUAL DIFFERENTIATION OF THE AFRICAN CLAWED FROG (*XENOPUS LAEVIS*)

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In a GLP-compliant study, commissioned by the Bisphenol A Global Industry Group, we investigated the effect of larval exposure to Bisphenol A (BPA) on growth, development and sexual differentiation in *Xenopus laevis*. Larvae were maintained in flow-through conditions at 22 ± 1°C and exposed to BPA at nominal concentrations of 1.0, 2.3, 10, 23, 100, 500 $\mu g\ l^{-1}$, from developmental stages 43/45 (beginning of independent feeding) through to 66 (completion of metamorphosis). Each test concentration, plus dilution water control (DWC) and positive control (PC, 17β-estradiol, 2.7 $\mu g\ l^{-1}$) employed 4 replicate test vessels with a starting density of 40 larvae/tank. Individual froglets were removed from test vessels upon reaching stage 66, and the study was terminated at 90 days. Froglets were dissected and sex was determined by inspection of gross gonadal morphology. Test concentrations of BPA had no effect on survival, growth, developmental stage distributions at exposure days 32 and 62, or mean time to completion of metamorphosis, when compared to DWC. Analysis of post-metamorphic sex ratio by G-test indicated no significant deviations from expected (50:50) sex ratio, in the DWC or any of the BPA test concentrations. In contrast, sex ratio in the PC (31% male, replicates pooled) deviated significantly from expected, and indicated that larvae had been feminised by exposure to the natural estrogen, 17β-estradiol. Exposure to BPA in the concentration range 1.0 - 500 $\mu g\ l^{-1}$ in flow-through conditions had no observable effect on larval growth, development or sexual differentiation (as determined by gross gonadal morphology) in this study.

PROSTATIC GROWTH RESPONSE IN SPRAGUE-DAWLEY AND FISHER 344 RATS TO NEONATAL EXPOSURE TO DIFFERENT CONCENTRATIONS OF ESTRADIOL BENZOATE

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Brief exposure of rats to high doses of natural estrogens early in life results in permanent alterations of the prostate gland, which include differentiation defects, altered gene expression and dysplasia with aging. Whether exposure to estrogen below the no-observed-adverse-effect-levels (NOAEL) causes similar or other effects is being controversially discussed. Possible variables making the assessment of the low-dose effect difficult include genetic differences between animal species and strains predisposing for differential responses to estrogen exposure. It was the objective of the current study to determine the dose-response of prostatic growth to neonatal exposure to natural estogen in two different rat strains, the Sprague-Dawley (SD) and Fisher 344 (F344) rats. SD rats were treated on postnatal days (PND) 1, 3 and 5 with a 7-log range of doses (0.0001 to 100.0 μg/injection) of β-estradiol-3-benzoate (EB) by subcutaneous injections in 25 μl of peanut-oil (*Arachis*) as vehicle. Animals were sacrificed on PND 35 and 90, the prostates were microdissected, weighed and frozen for immunohistochemistry. F344 rats were neonatally exposed on PND 1, 3, and 5 to 0.001, 0.1 or 10.0 μg/injection EB, respectively, euthanized on PND 90, and organs of interest removed. Pubertal markers (i.e. preputial separation, hepatic testosterones biotransformation enzymes) were monitored and measured. On PND 35, ventral, lateral, and dorsal prostate weights of SD rats treated with low doses of EB (0.001 μg/injection) were increased, while high doses (10, 100 μg/injection) reduced ventral prostate weights. On PND 90, no low dose effect was observed and only high-dose suppression of prostate sizes were found. Like in SD rats, prostate weights of low-dosed F344 rats did not display significant differences on PND 90. However, high dose treatment cause greater size reductions and differentiation effects than in SD rats. Likewise, responses of other steroid-sensitive reproductive organs (e.g., testes, epididymes, seminal vesicles) were greater than in the SD rat. Our results suggest that low doses of natural estrogens can cause increased prostate sizes around puberty, but not permanently into adult age. They furthermore confirm previous reports that F344 rats are more sensitive to estrogen treatment than SD rats, and that they therefore may be the preferential *in vivo* model for assessing effects of weak synthetic estrogens on the development of the male reproductive tract.

BISPHENOLS INTERACTIONS IN THE ESTROGEN RECEPTOR SIGNAL TRANSDUCTION PATHWAY

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Bisphenols and stilbenes demonstrated their estrogenicity *in vivo* in 1936 when it was reported the increase in uterine weight in rats after treatment with compounds containing two phenolic rings with hydroxyl groups in *para* positions. The stilbene diethylstilbestrol had pharmaceutical utility as a synthetic estrogen and bisphenols occupy a predominant position in the plastic industry. We have compared estradiol and different bisphenols (A basic bis-hydroxyphenyl structure to which we added to the central carbon alkanes—ethane to heptane—, ketone, propanol, perfluoropropane and methyphenol) in the pathway of estrogen receptor signal transduction in MCF-7 cells: i) Induction of mRNA of pS2, ii) Induction of the luciferase reporter gene placed under an estrogen-responsive element (ERE) in the MVLN cell line derived from MCF-7 cells, iii) Quantification of the estrogen induced progesterone receptor, iv) Quatification of the secreted pS2 protein, and v) Cell proliferation in the E-Screen bioassay. All the bisphenols tested elicited a full agonist response in all the assays. However some differences were found among: i) The concentration needed to induce a full estrogenic response (Potency), ii) The response range observed for estradiol and the bisphenols (Efficiency), and iii) The amplitude of response enable differentiation between an agonist, partial agonist and nil effect. This study demonstrates that bisphenols exerted estrogenic responses at different levels in the estrogen receptor transduction signal pathway but at higher concentrations than endogenous estrogens. The proliferation bioassay (E-Screen) was the most sensitive and effective among the four assays used.

MOLECULAR ASPECTS OF LOW AND HIGH DOSE BISPHENOL A PRENATAL EXPOSURE

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Bisphenol A (BPA) is among those industrial compounds which generate concern over adverse effects of environmental estrogens on wildlife and humans. BPA binds to the estrogen receptor alpha (ERα) and seems to be weakly estrogenic compared to 17β-estradiol (E2). In contrast, other data suggest that BPA has dose-dependent anti-estrogen activities.

Female offspring were exposed *in utero* on gestation days 6 through 21 to either 0.1 or 50 mg / kg / d BPA and 0.2 mg / kg/ d (E2). Histological analyses showed striking morphological changes in the differentiation, proliferation and cornification of the vagina from BPA treated animals. We, therefore, investigated the expression of ERα and estrogen receptor beta (ERβ) variants in the vagina of rat offspring during the estrous cycle at both molecular and protein levels using Western-Blot and RT-PCR analyses. For the first time, we demonstrate that the full-length ERα variant is not expressed during estrus at the protein level in the vagina of female offspring exposed prenatally to either dose of BPA compared to the control group. However, ERα-expression does not differ between the control group and the BPA exposed offspring during the diestrus stage. Furthermore, we could detect the expression of TERP-1 in the vagina of rat offspring. TERP-1,a truncated estrogen receptor product with functional activity is highly expressed in the diestrus stage, whereas its expression is decreased or absent in the estrus stage. Offspring exposed to E2 during gestation showed either a decreased or no expression of ERα in their vagina during the estrus stage. In addition we could not observe ERβ-expression at the protein level in the rat vaginal tissue from any group.

In conclusion this is the first finding which demonstrates that BPA affects the expression of ERα *in vivo* following *in utero* exposure of rats. Estrous cycle-dependent estrogen receptor expression is important for growth, differentiation and cornification of the vaginal epithelial cells. We therefore suggest that the loss of ERα expression in the vagina during the estrus stage leads to our previous morphological findings. The role of TERP-1-expression in rat remains unclear and has to be investigated in greater detail.

BISPHENOL A CONCENTRATION IN HUMAN UMBILICAL CORD BLOOD AND PLACENTA

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Bisphenol A (BPA) is an industrial compound highly employed in the manufacture of epoxy, polyester-styrene and polycarbonate resins with distinct physicochemical characteristics. These resins have uses in human health applications. When the polymer is in contact with food, especially at high temperatures (e.g., heated processed canned food or microwaved food) the ability of BPA to migrate from the polymer to the food has been described. Many scientists and regulatory agencies are concerned about the environmental effects of BPA. Furthermore, it is suggested that the amounts of BPA to which we are exposed could alter the reproductive organs of developing rodents. The EU Commission established a specific migration limit for BPA of 3 mg per kg because of fears that harmful quantities of BPA could leach into food.

Until now, there is little known regarding the extent of exposure of human pregnant mothers and their fetuses to BPA. Therefore, we established an analytical method for determining parent BPA in the placenta and plasma of human mothers and fetuses. Blood was taken from mothers (MB) during and after pregnancy. Human umbilical cord blood (CB) was taken from the umbilical vein after the placenta was expelled. Plasma was obtained by centrifugation of CB and MB samples which were collected by drainage of blood into sterile tubes not leaching BPA into the blood or plasma sample. Placental tissue was homogenized and solubilized.

After assessing analytical reagents from different suppliers, altering the way of derivatization and introducing special methods to homogenize placental tissue, we developed a chemical derivatization-gas chromatography (GC) / mass spectrometry (MS) method to analyze parent BPA at concentrations less than 1 μg / ml. For the first time, we demonstrate that parent BPA exists at concentrations between 3 and 100 ng / g of human placenta.

According to research conducted in Japan by O. Takahashi et al. (Environ. Health Perspect. 2000 Oct;108(10):931–935) on rats showing that the placenta does not serve as a barrier to transmission of BPA, we suggest that parent BPA can be transferred from the human mother to the fetus via the placenta. Although parent BPA is rapidly eliminated by first-pass metabolism following oral

administration, parent BPA can be found in human placental tissue following normal food consumption.

ENDOCRINE DISRUPTION: CURRENT STRATEGIES IN THE US

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The endocrine disruptor hypothesis was originally inspired by reports of apparent geographic and temporal trends in adverse health effects observed in wildlife and human populations. These ecological reports were correlated with trends in production, use, or environmental levels of persistent bioaccumulated halogenated organic compounds. Some of these trends, such as apparent decreases in sperm count, are more complex and less generalized than first reported. Studies associating cancer risks with exposure to these chemicals have been contradictory or inconclusive. The suggestion that inferences can be readily drawn from wildlife to humans may not hold for all endpoints, particularly those related to development. Some of the observed effects of EDCs in nonmammalian species, such as sex reversal, cannot occur in mammals, while effects such as reproductive failure of populations, are unlikely to be associated with environmental exposures. Some relatively sensitive effects of EDCs in mammals, such as the vaginal dysmorphogenesis induced by TCDD, may be species-specific.

Bisphenol A (BPA) represents the category of less persistent but ubiquitous compounds present in many consumer products. Studies of its effects on mammalian development support the current hypothesis that development may be most sensitive to endocrine disrupting chemicals, particularly during periods when physiological systems are organized by maturational changes in endogenous hormone exposure. Research in our laboratory suggests that BPA has a different spectrum of estrogenic activity in vivo than does DES or chlordecone. BPA, like DES and CD, reduced relative anogenital distance in female rats after prenatal exposure; however, BPA did not reduce prostate or seminal vesicle weight in contrast to DES. While CD and DES had a transient effect to increase the size of the sexually dimorphic nucleus, BPA was not associated with increased size of this nucleus as compared to control females.

These results suggest that it is not clear how best to develop tests to identify potential EDCs and how to interpret the results of these tests. In vitro systems may not predict in vivo responses. Animal studies may not provide clear indicators for epidemiological studies. EDCs like BPA may be selective endocrine modulators, exerting agonist-type effects in some tissues at some stages of development, and no response in other tissues or at other stages.

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PROLIFERATIVE EFFECTS OF *IN UTERO* EXPOSURE TO BISPHENOL A ON MAMMARY GLAND DEVELOPMENT

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Exposure to estrogens throughout life has been established as a major risk factor for the development of breast cancer. Recent epidemiological and experimental studies reveal that exposure to high levels of estrogens during intrauterine development of the fetus correlates with an increased incidence of breast cancer in the adult. The introduction of estrogenic chemicals into the environment therefore poses a very real threat to human health, particularly if exposure occurs during critical periods of fetal histogenesis and organogenesis. Bisphenol A (BPA) is a monomer used in the manufacture of resins (linings of tin cans, dental sealants) and plastics (babies formula bottles, food storage containers) that has been shown both *in vitro* and *in vivo* to exhibit an estrogenic nature. Under normal conditions of use, this chemical leaches from such products into foods, beverages and saliva therefore causing potential for fetal exposure. The aim of this study was to determine the effects of *in utero* exposure to environmentally relevant concentrations of BPA on the development of the mammary gland and to establish tissue changes that may precede carcinogenesis. The doses were arbitrarily chosen to be 4,000 and 400-fold lower than those needed to induce an uterotrophic response in the immature CD-1 mouse. Pregnant CD-1 mice were implanted with subcutaneous pumps that delivered either DMSO vehicle, 25 μg/kg body weight BPA, or 250 μg/kg body weight BPA on day 8 of gestation. The genital tract and mammary glands were collected from female offspring at 10 days, 1 and 6 months of age. Among the pathologies seen in these tissues, the mammary gland showed the most striking histoarchitectural changes. No differences between control and BPA-treated groups were apparent at birth and 10 days of age. At puberty there were differences in ductal elongation between the groups. At 6 months of age, quantitative analysis of whole-mounted tissues revealed an increase in the percentage of ducts, terminal ducts (TD), terminal end buds (TEB) and alveolar buds (AB) in both BPA-treated groups. These findings demonstrate that environmentally relevant levels of BPA, when administered during intrauterine development, can induce dramatic proliferative and maturational changes in mammary gland tissue that are consistent with estrogenic activity. Further, the ability of this chemical to alter tissue organization highlights the importance of discerning the role that environmental estrogens may be playing in the increased incidence of breast cancer seen over the last 30 years.

A MULTI-GENERATION STUDY OF THE EFFECTS OF BISPHENOL-A ON THE FATHEAD MINNOW (PIMEPHALES PROMELAS)

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To investigate the possible effects of long-term exposure to Bisphenol-A (BPA), a multi-generation study was conducted, in which 3 generations of the fathead minnow were exposed to water concentrations of BPA covering the range 1 to 1280 μg/litre. Exposure to BPA began when the FO generation fish were subadults, and the experiment lasted a total of 431 days, so that the F1

generation fish were exposed for all of their lives, and the F2 generation fish until 60 days post-hatch. The two highest concentrations of BPA (640 and 1280 μg/litre) inhibited growth of both the FO and F1 generations. Vitellogenin concentrations, which are indicative of exposure to an oestrogenic chemical, were markedly elevated in males exposed to concentrations of BPA at and above 160 μg/litre. Female fish, which had much higher vitellogenin concentrations initially, showed much less of a response to BPA. Gonadal growth was inhibited in both sexes by the two highest concentrations of BPA. The most sensitive parameter affected by BPA was spermatogenesis, which was inhibited at concentrations at and above 16 μg/litre. However, reproductive output, assessed by measuring egg production and hatchability, was affected only at the two highest concentrations of BPA. Essentially the same effects occurred in both the FO and F1 generations. However, the F1 generation appeared somewhat more sensitive (lower concentrations produced significant effects), but this comparison was complicated by the fact that the FO generation fish were exposed to BPA for only the second half of their lives, whereas the F1 generation fish were exposed throughout their entire lives. The results demonstrate that BPA acts as a weak oestrogen to fish when administered via the water, with effects on breeding at and above a concentration of 640 μg/litre.

THE EFFECTS OF LOW AND HIGH DOSE *IN UTERO* EXPOSURE TO BISPHENOL A ON THE REPRODUCTIVE SYSTEM OF FEMALE RAT OFFSPRING

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The endocrine active substance, bisphenol A (BPA), is ubiquitously employed in food and beverage containers and is also a component of some dental preparations. Concern exists whether the potential effects of BPA on the fetus, particularly at doses below the currently accepted NOEL (50 mg/kg/d) for this compound, have been sufficiently characterized. We examined the effects of prenatal exposure to BPA (0.02, 0.1 and 50 mg/kg/d) or ethinyl estradiol (EE) (0.02 and 0.2 mg/kg/d) per gavage on gestation days 6 through 21 on the reproductive system of female Sprague Dawley rat offspring. The control gravid dams received a 2% cornstarch suspension (1.0 ml/100 g BW). Beginning on postnatal day 35, the female offspring were examined for vaginal opening, an accepted landmark of sexual maturation. At approximately 13 weeks of age, vaginal cytology was performed for 21 consecutive days to evaluate the estrous cycle. The females were sacrificed either in estrus or diestrus at approximately 16 weeks of age to evaluate reproductive organ weights and diestrus progesterone concentrations. Sexual maturation was significantly delayed following exposure to BPA and EE with the greatest delays observed in the low dose groups. An alteration in the reproductive cycle in the form of extended estrus phases was detected in all treatment groups. The mean progesterone concentration in diestrus was significantly decreased in the low dose (0.02) BPA and EE groups, while it was significantly increased in the high dose BPA (50) group. Prenatal exposure to BPA at doses corresponding to, and below its NOEL, alters postnatal reproductive system function in the female Sprague Dawley rat. These changes did not occur in a classical dose-dependent fashion indicating that current toxicological assessment guidelines for evaluating endocrine active compounds need to be reassessed. This work was supported by a grant from the BMBF (07HORO1/7).

THREE-GENERATION REPRODUCTIVE TOXICITY STUDY OF BISPHENOL A (BPA) ADMINISTERED IN THE DIET TO CD® (SPRAGUE-DAWLEY) RATS

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BPA is a high production volume chemical used principally as a monomer in the manufacture of polycarbonate plastics and epoxy resins. It can produce some "estrogen-like" effects at high gavage and parenteral doses; initial studies (not replicated) have reported that it is also active at low doses. Therefore, BPA (>99.5% pure) was evaluated in a multigeneration reproductive toxicity study (under U.S. EPA OPPTS 1998 testing guidelines with additions and in compliance with EPA GLPs) at dietary concentrations of 0, 0.015, 0.3, 4.5, 75, 750, and 7500 ppm, available *ad libitum* to 30 animals/sex/dose for three generations, one litter/generation, through F3 adult offspring. Adult systemic toxicity was present at 750 ppm (approximately 50 mg BPA/kg/day) and 7500 ppm (500 mg/kg/day) for all adult generations, expressed as reduced body weights (BW) and BW gains during prebreed (both sexes) and gestation and lactation, reduced absolute and increased relative weanling and adult organ weights, and increased incidences of renal tubular degeneration and chronic hepatic inflammation only at 7500 ppm in F0, F1, and F2 (but not F3) females. Reproductive organ histopathology and function were not affected in either sex in any generation. Paired ovarian weights and total and live pups/litter on pnd 0 were decreased at 7500 ppm (in the absence of any increase in prenatal postimplantation loss) in the presence of substantial maternal toxicity in all generations. There were no treatment-related effects in any generation on parental or offspring mortality, clinical signs of toxicity, or on mating, fertility, or gestational indices, ovarian primordial follicle counts, estrous cyclicity, precoital interval, gestational length, offspring sex ratios or survival, on nipple/areolae retention in preweanling F1, F2, or F3 males, on adult F0, F1, F2, and F3 male epididymal sperm number, motility, or morphology, on adult F0, F1, F2, and F3 male testicular homogenization-resistant spermatid head counts, daily sperm production (DSP), or efficiency of DSP. At 7500 ppm, there were also delays in acquisition of puberty (vaginal patency and preputial separation) in F1, F2, and F3 offspring of both sexes, associated with reduced BW, which began during lactation (pnd 7) and continued through adulthood. Anogenital distance on pnd 0 was unaffected for F2 and F3 males and F3 females, and increased (by 0.03–0.05 mm) in F2 females at 0.015, 0.3, 4.5, and 750 ppm, in the absence of any effects on pnd 0 pup body weights, with no

dose-response pattern, and no developmental or reproductive sequela. There were no consistent or persistent treatment-related effects of dietary BPA exposure on any adult or offspring parameters at 0.015, 0.3, 4.5, or 75 ppm (equivalent to approximately 0.001, 0.02, 0.3, or 5 mg BPA/kg/day) in rats in this study.
In conclusion:

- The adult systemic toxicity NOAEL for both sexes was 75 ppm (approximately 5 mg BPA/kg/day)
- The reproductive and postnatal toxicity NOAELs were 750 ppm (approximately 50 mg BPA/kg/day)
- There were no treatment-related low dose effects.
- There was no evidence of a non-monotonic dose response
- The absence of treatment-related low dose effects in this study are consistent with the findings from a two-generation reproduction study in rats recently completed by the Chemical Compound Safety Research Institute, Hokkaido, Japan, in which CD® rats, 25/sex/group, were gavaged daily with 0, 0.2, 2, 20, 0r 200 μg BPA/kg/day; there were no treatment-related effects of BPA on any parameters examined at any dose.

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HIGH SENSITIVITY OF THE FETAL PROSTATE TO ENDOGENOUS AND ENVIRONMENTAL ESTROGENS

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Estrogens are now known to modulate development of both the male and female reproductive organs, although estrogen is not essential for development of these organs. We have previously shown in both rats and mice that a very small difference in serum estradiol in male fetuses is correlated with prostate size during fetal life and in adulthood. We extended these findings by examining male CF-1 mice that were identified at Cesarean delivery as being located between 2 females (2F, with elevated estradiol) or two males (2M, with elevated testosterone). When 3 months old, the 2F and 2M males (5–7/group) were castrated and implanted with a Silastic capsule containing either testosterone (T) or 5a-dihydrotestosterone (DHT). When examined 3 weeks later, 2F males had larger prostates than 2M males when treated with T or DHT, which shows that the effect of IUP on the prostate is likely mediated by the difference in androgen receptors and not 5a-reductase. In other studies male CF-1 mouse fetuses were exposed via administration to the mother to a very low dose of estradiol (E), ethinyl estradiol (EE, 0.1 μg/kg/day) or diethylstilbestrol (DES, 0.1 μg/kg/day), and prostate size in adulthood was increased. In addition, there was a permanent increase in prostatic androgen receptors. We recently examined the prostate of male CD-1 mice on the day of birth after prenatal exposure to these same doses of EE and DES. Prostates were examined using computer assisted 3-D reconstruction. There was a significant increase in the number of prostate glands (by about 40%) as well as overall prostate size (by about 80%) relative to controls. Fetal exposure to a very low dose (10 μg/kg/day) of the plastic monomer bisphenol A produced all of the effects (at the same magnitude) on the developing prostate in both CF-1 and CD-1 mice as were observed with E, EE and DES. This finding demonstrates that in the developing prostate, bisphenol A is a full estrogen agonist in CF-1 and CD-1 mice.
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ROUTE-DEPENDENCY IN THE BIOAVAILABILITY OF BISPHENOL A (BPA) IN FISCHER 344 RATS AND DIFFERENTIAL METABOLISM OF BPA IN PRIMARY CULTURED HEPATOCYTES FROM RATS, MICE AND HUMANS

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The objectives of these studies were to determine if a route-dependency exists in the pharmacokinetics and metabolism of ^{14}C-BPA and to determine the metabolism of BPA in monolayers of primary hepatocytes from rats, mice and humans. Analysis of metabolites was by radiochemical HPLC, with identification of plasma metabolites by LC/-ESI/MS, and *in vitro* metabolites by LC/MS/MS. *In vivo*, the relative bioavailability of BPA and plasma radioactivity was markedly lower following oral administration as compared to subcutaneous (sc) or intraperitoneal (ip) administration. The major fraction of plasma radioactivity following oral dosing was comprised of the monoglucuronide conjugate of BPA (68–100%). Up to four additional unidentified metabolites were present only in the plasma of animals dosed ip or sc; one of these was tentatively identified as the monosulfate conjugate of BPA. *In vitro*, the monoglucuronide was the major metabolite in all species/strains, except in F344 rats where a mixed sulfate/glucuronide conjugate predominated. The initial rates of metabolism in hepatocytes followed the order of mice > rats > humans, however when extrapolated to the whole liver, the differences in the number of cells/gm of liver reverse this relationship to humans > rats > mice. By 3 hrs, the rate of metabolism was similar in all species with BPA converted completely to conjugates. In conclusion, these *in vivo* results demonstrated that oral administration resulted in very low bioavailability of BPA in rats; this result is also expected in humans due to the differences in liver mass and the similarity in the *in vitro* metabolic profiles across species.

LACK OF EFFECTS OF PERINATAL EXPOSURE TO LOW DOSES OF BISPHENOL A ON MALE RAT OFFSPRING VENTRAL PROSTATE GLANDS

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A replicate block design study using one or two pups/sex/litter as representative of biological events in same sex littermates revealed an apparent increase in ventral prostate (VP) tissue fresh weights of 6 months old male F1 Sprague–Dawley (SD) offspring. Their mothers were exposed to a wide dose range of BPA via drinking water (Toxicol. Sci. 54, Suppl., 256A, 2000). The VP weights were highly variable, an observation confirmed by other CIIT studies with full litter complements. Very low VP weights were particularly striking in the one pup/litter control animals which raised concerns about sample size. A follow-up

evaluation driven by statistical power considerations was conducted to reach an unequivocal decision as to whether or not BPA-induced effects could be ascertained. Maternal BPA exposure (n = 20/dose group) occurred via drinking water from gestation day 2 through weaning on postnatal day (PND) 21 with estimated daily intakes of 0, ~1 μg, ~70–120 μg and ~7–12 mg/kg/day. Four randomly selected males were then held with no further BPA exposure until 6 months old. The male reproductive tract organs, including sexual accessory glands, were then collected for fresh weight determinations. The prostate was divided into its ventral and dorsolateral lobes and fixed for histological examination. Compared to concurrent controls no significant BPA-induced changes occurred in any of the end points examined. No differences in prostate micromorphology were detectable. The data revealed that protracted maternal ingestion of very low to moderate levels of BPA does not affect the VP fresh weights of male rat offspring.

WHY BISPHENOL A IS WEAK IN THE ADULT YET STRONG IN THE FETUS

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Bisphenol A (BPA) is widely used in polycarbonates and epoxy resins. Since BPA leaches from these sources, human exposures may be significant. In rodents, fetal exposure to BPA at doses below those that stimulate adult estrogenic responses can enlarge the prostate and accelerate female puberty. To determine circulating levels of BPA present after developmentally-active low doses, pregnant mice were fed tritium-labeled BPA in oil at 2 or 20 ug/kg body weight, either once or multiple times (4 to 8 daily doses), ending on day 18 of gestation. While maternal serum BPA declined rapidly over the first 3 hours, the concentration then stabilized at a level maintained past 24 hours. This stable circulating concentration was increased following multiple daily doses in pregnant females, although not in nonpregnant females. Twenty-four hours after the last of 7–8 daily doses, serum BPA averaged 100 pg/ml (0.44 nM). However, substantial individual variation in BPA levels was observed, and may identify a sensitive subpopulation particularly susceptible to endocrine disruption by BPA. Bioaccumulation of BPA in maternal circulation, at a time when the fetus is most vulnerable to permanent, non-reversible changes, may represent a significant source of exposure to the fetus and may contribute to the high sensitivity of the fetus to BPA. Supported by UMC VMFC0018 and NIH ES08293.

BISPHENOL A CONCENTRATIONS IN THE AQUATIC ENVIRONMENT

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For evaluating the environmental safety of chemicals, knowledge of exposure concentrations is a crucial prerequisite beside effects information. Therefore, BPA was analyzed in selected surface waters, sewage effluents and sediments in Germany and in tissue samples from the German federal environmental specimen bank, sampled between 1985 and 1996.

In surface waters, BPA was found in 43 out of 52 samples with a median concentration of 23 ng/L (max. 229 ng/L) and in 35 out of 40 sewage effluents with a median concentration of 62 ng/L (max. 702 ng/L). Sediment concentrations were between 0.75 and 23 mg BPA/kg dry matter. Results of other groups confirm, that BPA is present in relatively low concentrations up to 410 ng/L in surface waters, except some hot spots, and in sewage effluents up to 700 ng/L.

For analyzing BPA in tissue samples, the method was adapted and optimized (limit of determination LOD: 1 μg/kg fresh weight). The BPA content in the examined tissue samples was generally low. In zebra mussel, concentrations were primarily between 1–2.5 μg/kg and below LOD in bream muscles. In samples from the marine environment, BPA concentrations were 1–2 μg/kg in brown algae, approx. 2.5 μg/kg in seagull eggs and eel pout muscles, and below LOD for mussels.

Based on so far published studies, endocrine disrupting effects in aquatic organisms had been observed at concentrations in the μg/L range.

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EFFECTS OF XENOESTROGENS ON THE TOTAL SERTOLI CELL NUMBER—DETERMINATION OF THE VALIDITY OF UNBIASED STEREOLOGY

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To assess effects of Bisphenol A on the total Sertoli cell number in the testis the optical disector is an ideal tool, but the observation needs to be unbiased and valid. This method involves the physical dissection of the testis. A number of thick sections per fragment are cut and evaluated by focusing through several planes along the z-axis. The observed fields are chosen by systematic randomisation. The scores allow calculation and quantification of the cells of a defined volume by nearly complete preservation of the structural integrity. A number of fields is sampled in every section. Intersectional and intrasectional variances, the number of zero-counts without the observed cell type and the number of counted volumes per section can influence the results of the scores. To validate the method testis fragments have been sampled for a group of 5 control animals and 7 animals treated with Bisphenol A perinatally. For each fragment between 5 and 60 volumes per slide were counted and the total cell numbers, the coefficient of variation (cv) and the amount of zero counts were calculated and compared. The results of this study show that at least 40 volumes per slide have to be counted to calculate valid data. The results vary if a smaller number of counted volumes is used for calculation. The validity of the procedure was confirmed by the fact that neither intrafragmental nor intrasectional differences were seen, but some intersectional variance indicate that the fragments are not always homogeneous (gsf 07HOR 03/9).

IDENTIFICATION OF BISPHENOL-A CHLORODERIVATIVES IN DRINKING CHLORINATED WATER. ESTROGENIC PROPERTIES

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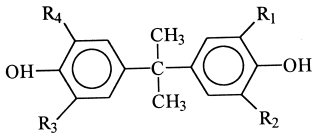

Fig. 1. $R_1 = R_2 = R_3 = R_4 = H$; (2) $R_1 = Cl$, $R_2 = R_3 = R_4 = H$; (3) $R_1 = R_3 = Cl$, $R_2 = R_4 = H$; (4) $R_1 = R_2 = R_3 = Cl$, $R_4 = H$; (5) $R_1 = R_2 = R_3 = R_4 = Cl$

Bisphenol A (BPA) is a estrogenic monomer widely used in the manufacture of epoxy and phenolic resins, polycarbonates, polyacrilates and corrosion-resistant unsaturated polyester-styrene resins. It may be found in a diverse range of products including the interior coatings of food cans and filters, water containers, dental composites and sealants. Here the chlorination reaction of BPA in chlorinated tap water is studied using Spectrofluorimetry and GC-MS analysis. The different chlorinated BPA derivatives (2–5) were synthesized, isolated and identified. The structure of these compounds were univocally established by Ms, ^{1}H-NMR, and ^{13}C–NMR spectroscopy (Figure). The influence of different experimental parameters (pH, reaction time, chlorine concentration and presence of cationic and anionic species) is also established. The present study also investigates the estrogenicity of these chlorinated bisphenols in the E-Screen bioassay. The proliferative effect (PE) of chlorinated bisphenols was significantly greater than 1 for for all the compounds tested. Chlorinated BPA with the lowest degree of chlorination showed highest estrogenic potencies. In comparison with the PE of estradiol, all the positive compounds showed a full to partial agonistic response that produced cell yields ranging from 85% of estradiol-induced yield for bisphenol-A to 30% for tetrachlorine bisphenol-A derivative (5).