Plastics Additives

Katherine Unruhe

2012

Plastics Additives Unit Plan

- **Day 1: Plastics Lecture and Polymer making activity**
  - Warm-Up (10 min): Students will see a plastic water bottle at the front of the classroom. Students will list everything that they think has gone into the production of the plastic bottle. The teacher will then list everything students have said on the board.
  - Plastics: The Basics (20 min): Teacher will provide background information about the sources of plastic and the chemical structure of plastic. Materials: Plastics (PowerPoint)
  - Polymer making activity (30 min): Students will make bouncy balls from ethanol and sodium silicate. They will answer questions about the chemical structure of the resulting polymer. Afterwards the teacher will lead a review of the lab, making sure that students understand that polymers are strong and flexible because of the long interlocking chains that form, but that different polymers have different properties, which can be changed with additives. Materials: Polymer Making Activity
  - A quick look at BPA (30 min): Students read Scientific American Article about Bisphenol-A in plastics. Students then form groups and discuss:
    - Why is there so much controversy over BPA?
    - Do you think that there is cause for concern?
    - If you were a regulator, would you restrict the use of BPA in plastic products with the information that you currently have? What further studies would you like to commission?

    Materials: Just How Harmful Are BisphenolA Plastics
- **Day 2: The Endocrine System**
  - Warm-Up (10 min): Students list 3 things they know or think they know about the signaling process that tells teenage bodies to undergo puberty. The teacher lists students' answers on the board, and discusses them briefly.
  - Presentation: The Basics of the Endocrine System (15 min): Teacher will provide basic information about the endocrine system. Materials: The Endocrine System (PowerPoint)
  - Student Research (25 min): In groups students are assigned hormones and must find out the signaling pathway of the hormone, how it is transported through the body, its negative feedback loop, and the effect it has on the body.
  - Presentations (30 min): Teacher will prep for student presentations by posting the organs of the endocrine system on the wall around the room. Student groups are then given five minutes to present the basic information about their hormones by using signs and moving between

the different organs in the body. Materials: last slide of The Endocrine System (PowerPoint) and Hormone Presentations Rubric

- Fun Quiz (10 min): Students match fun facts with the hormones from the student presentations. Teacher reviews immediately afterwards. Materials: The Hormone Quiz

- **Day 3: A Regulatory Discussion**
  - Warm-Up (10 min): Three questions from the previous days hormone quiz are on the board. Students write down the name of each hormone. Teacher briefly goes over the answers.
  - In the News (15 min): Students read a Scientific American article about mixtures of chemicals in the natural environment, including mixtures of phthalates. Students then write down answers to the following questions before discussing them in small groups:
    - In the 6th paragraph, Marian Stanley talks about "endpoints that may not have any biological relevance." Do you consider this a valid argument? Why or why not?
    - What are some of the difficulties of testing for the effect of mixtures? Do you think testing of this type should be mandatory? If so, in what situations?

    Materials: Mixing it Up
  - Debate Prep (30 min): The teacher assigns each student a jig-saw group number/letter combination. Students first form into their number groups, and the teacher assigns each of these groups one of the phthalate studies from the handout. Students complete the questions in the handout and discuss how best to present the material to their peers. Materials: Phthalate Studies Activity, selection of Phthalate studies
  - Jig-Saw Share (20 min): Students now form groups by their letter. Each letter group should have at least one person from each numbered group. One student at a time shares the basic information that they got from their study, while other students ask questions and fill in their tables. Materials: Phthalate Studies Chart
  - Class Discussion (15 min): Students discuss the following questions:
    - If you were on a regulatory panel, what additional information or studies would you request in order to make a decision about the safety of phthalates?
    - Do you think innocent until proven guilty is good in regulatory procedures as well as judicial ones?
    - If animals can still reproduce, should we be concerned with small reproductive deformities?
  - Filler Activity: Students read about the struggle of the Aveda company to produce environmentally responsible products. Students then discuss in small groups possible regulatory changes that would make it easier for companies to know what is going into their products. Materials: Aveda Case Study

- **Day 4: Student Research**
    - Warm-Up (10 min): Students write down the signaling pathway for one hormone. After 5 minutes, the teacher asks for student volunteers to share the signaling pathway that they wrote down.
    - Project Presentation (5 minutes): Teacher explains the research project that students will be working on for the rest of the class period, and finishing for homework. Materials: Plastic Additives Research Activity
    - Student Research (50 min): Students use computers and books to research their chosen plastic additive.
    - Student Presentation Formation (25 min): Students begin to put together a presentation for the class using PowerPoint or Presi. Whatever is not finished in class will be finished for homework.
- **Day 5: Presentations**
    - Presentations (70 min): Students present using PowerPoint or Presis. While their classmates are presenting, students will write down general discussion questions brought up by the presentations. Some general topics to focus them on: Common themes, future direction for regulatory rules. Materials: Plastics Additive Presentation Rubric
    - Class Discussion (20 min): Students split into small groups, different from their presentation groups, and discuss student questions for 10 minutes. Students then come back together as a class and share the most fruitful part of their small group discussion, and end by posing a large-group question.

Plastics- The Teacher Guide

Plastics have become ubiquitous in today's world. If you count how many different plastic items you touch during a day, most people will arrive at a number well over 100. Plastics come in a huge variety; some are flexible, some are rigid, some clear, and some colored. These different plastics not only have very different properties, but also have very different chemical make-ups and different chemical additives as well. You will find below some basic information about plastics, as well as more specific information on plastics that commonly contain chemical additives that may well be endocrine disruptors.

Plastic Basics

All plastics are polymers, or long chains with smaller repetitive parts that we call monomers. For instance, polyvinyl chloride (PVC) plastics are made when smaller vinyl chloride molecules link together to form long chains. These long chains are huge in comparison with simple molecules such as water. In terms of size they are more similar to the proteins naturally made in our bodies. In fact, proteins, along with cellulose in cell walls and DNA are all biosynthetic polymers. (polymer is Greek for "many parts")

Most plastics today are derived from oil or natural gas, but plastics can come from many different sources. The first commercial plastic was made from mixing cotton and acid, which created a hard, rigid plastic. Later, more efficient ways were found to make plastics from oil and natural gas. The backbone of plastic is usually made up of carbon, with some oxygen and hydrogen bonded to carbon at the backbone. Plastic can also be halogenated. If chloride is added, you get vinyl or PVC. If fluoride is added you get Teflon™. As both oil and natural gas are rich in hydrogen and carbon, they are easily made into plastic. To make plastics from natural gas, the gas is heated in columns. At different temperatures within the column, different types of plastic form (often differentiated by the different types of carbon rings that form).

Plastics only became popular consumer products in the 1940's during WWII. The war lead to shortages of many materials, which were then replaced with plastic as that could be manufactured cheaply on the home front. In recent years plastic use has grown exponentially. The quantity of plastic produced in the first 10 years of the current century is likely close to the total amount of plastic produced during the last century according to a study by Richard Thompson and colleagues.

Some Common Plastic Types

Type of plastic	Where it's found	Notes
Nylon	Bristles on a toothbrush, pantyhose, parachute material, soft side of Velcro	

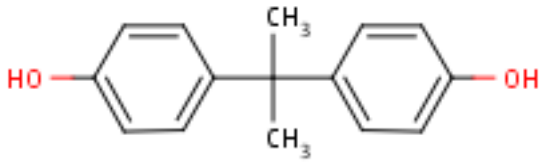
Acrylic	Paint	
Polyester	Clothing	May contain BPA
Polypropylene #5	Yogurt containers, microwavable dishes, disposable diapers, cars, bottle caps	This is a good alternative to PVC because it doesn't have chlorine in it's structure or need plastic additives
Polyethylene High density #2 Low density #4	High density- milk jugs, plastic bags, yogurt cups Low density- produce bags, food storage containers	
Acrylonitrile	Astroturf	
Celluloid	Original movie film (made from the cellulose in cotton)	
Polystyrene #6	Hangers, combs, other hard clear objects, Styrofoam™	Can leach styrene and alkylphenols
Polyethelene terephthalate (PET) #1	Flexible soda and water bottles, fiber for clothing and carpet	Can leach antimony and phthalates
Polyvinyl chloride (PVC and vinyl) #3	IV bags, tubing, shower curtains, floor tiles, upholstery, garden hoses, raincoats, wire sheathing, flip-flops, yoga mats	Can leach lead and phthalates
Polycarbonate #7 (#7 is a catch-all category for plastics, so while all polycarbonates are #7 plastics, some #7 plastics are not polycarbonates)	Hard water bottles	Can leach Bisphenol-A

Plastic Additives

Bisphenol-A (BPA)

BPA is found in polycarbonate plastics, a hard clear plastic found in water bottles, CDs, glasses, baby bottles and the lining of tin cans. BPA is also found in thermal receipt paper. When BPA-containing plastics are scratched, scuffed or exposed to high temperatures, the BPA is loosened from the polymer structure and can leach into whatever is inside the container. The US Center for Disease Control has found traces of BPA in 93% of all collected urine samples, with levels ranging from 33 to 80 nanograms per kilogram bodyweight. Children had the highest levels of BPA, and adolescents had the second highest levels.

BPA mimics the hormone estrogen by binding to the same receptor as natural estrogens, and can have different effects depending on the developmental stage of the person consuming BPA. In animals, BPA has been shown to cause breast cancer, heart disease, type 2 diabetes, obesity, hyperactivity and lower sperm counts. Like many endocrine disrupters, BPA is often not toxic at higher doses but is toxic at lower doses. Higher doses somehow shut off the effects seen at lower levels. The harmful (low) levels calculated from these animals studies (20 micrograms per kilogram) is 10 times below the common exposure level in the United States. In other words, adult Americans are exposed to levels of BPA (measured per kg bodyweight) at much higher levels than were found harmful to test animals such as mice and rats.
While there is still a lack of human studies on the effects of BPA, some recent studies have been troubling. One recent study by Joe M Braun of Harvard University suggests that prenatal exposure to BPA is connected to anxiety, depression and difficulty controlling behaviors in three-year olds (especially girls).
For those concerned with reducing BPA from their diet, one recent study suggests a "fresh foods" diet, which includes no canned or packaged food. BPA levels in study participants following the fresh foods diet dropped by 66%. Experts also suggest that clear plastic containers should never be micro-waved or used to store hot foods or liquids.

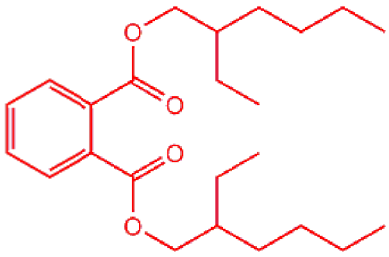


Chemical Structure of Bisphenol-A.

Phthalates

Phthalates are most commonly found in flexible forms of PVC plastic, but are also found in some PET plastics. Some examples of items that contain phthalates are plastics in shower curtains, wall paper, window blinds, floor tiles, upholstery, garden hoses, raincoats, sheathing on cables and wire, flip-flops, yoga mats and cling wrap. Phthalates are also found in cosmetics and nail polish. The main source of human exposure is believed to be fatty foods, which absorb the phthalates from the packaging or from some part of the manufacturing process. Phthalates, like fats, are chains of carbon. Since like dissolves like, phthalates can be thought of as having an affinity for fatty substances, and will leach into fatty substances more quickly than into other food types such as carbohydrates. Studies by the Center for Disease Control have shown that 80% of Americans have measurable quantities of phthalates in their bodies.
There are many different types of phthalates, with some being greater estrogen disrupters than others. To better understand the effect of phthalates on the human

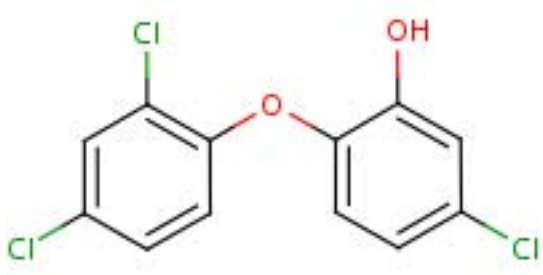
body we can look at one particular phthalate that is widely accepted as an endocrine disrupter; DEHP. When DEHP enters the body it is metabolized. This metabolite then signals the pituitary gland to stop making the hormone that signals the gonads (ovaries in women and testes in men) to make testosterone. Testosterone plays an important role in sexual differentiation, and this physiologic function seems be especially sensitive to effects from phthalate exposure.
Some of the first studies on phthalates were done on rats. When pregnant rats were exposed to phthalates the newborn rats had significant differences in the rate of undescended testes, as well as differences in anogenital distance and the location of the urethral opening on the penis. These conditions have collectively come to be known as phthalate syndrome in rats. Recent studies in humans have also shown this effect. One study by Shanna Swan of the University of Rochester found that women with higher levels of phthalates in their blood during pregnancy had children with symptoms echoing the phthalate syndrome found in rats, such as smaller penis size and undescended testes. In a follow-up of the same study testing the same children at age three, those whose mother's had high phthalate levels during pregnancy were less likely to engage in typically male play behavior. Currently, all human studies have had small numbers of participants, so more research is needed, but the results of the human and animal trials are certainly suggestive of a strong endocrine disruptive effect.



Chemical Structure of DEHP, a common phthalate.

Triclosan

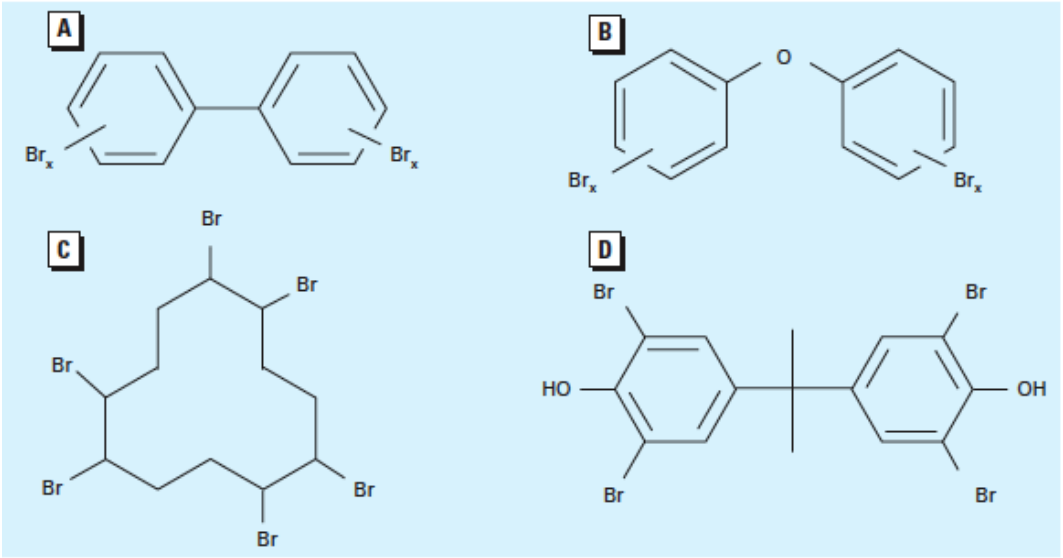
Triclosan is an antibacterial that has been added to many different types of plastics. It is also found in hand soaps, shampoo, and conditioner, and toothpaste to name just a few of its other uses. There have been some studies that connect triclosan with animal birth defects and cancer. As far as this author knows there are currently no studies showing the effects of triclosan in humans.
According to a CDC study triclosan is found at detectable levels in the urine of 75% of Americans. It is also known to be bio-accumulative, and is found in higher concentrations in fatty tissues such as breast tissue.



Chemical Structure of Triclosan.

Brominated Flame Retardants

Brominated Flame Retardants or BFRs are a little-researched plastic additive. As the name suggests they are added to plastics as well as to furniture, curtains, and other household items to prevent fires. When added reactively they are incorporated into the actual structure of the polymer. When added additively they are added in such a way that they are not covalently bound to the polymer structure of the plastic. It is thought that reactive BFRs are less likely to leach but both reactive and additive treated products have been shown to release BFRs. Current studies of cell cultures suggest that most toxic effects come from disruption of thyroid homeostasis. This is an endocrine disruption effect.



Chemical Structure of (A) PBBs, (B) PBDEs, (C) HBCD, and (D)TBBPA, all common Brominated Flame Retardants. Picture from the journal article *Brominated Flame Retardants: Cause for Concern?* by Linda S. Birnbaum and Daniele F. Staskal

Resources

Books
Our Stolen Future by Theo Colborn

RISE

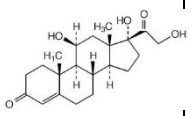
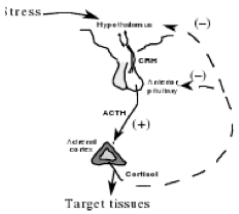
Plastic: A Toxic Love Story by Susan Freinkel
Paper or Plastic: Searching for Solutions to an Overpackaged World by Daniel Imhoff
Atlas of Plastics Additives: Analysis by Spectrometric Methods by D. Hummel
Exposed: The Toxic Chemistry of Everyday Products and What's at Stake for American Power by Mark Schapiro
Hormonal Chaos: The Scientific and Social Origins of the Environmental Endocrine Hypothesis by Sheldon Krimsky

Websites

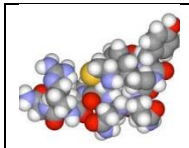
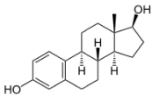
e.hormone.tulane.edu
http://e.hormone.tulane.edu/learning/images/Hormone-Docking-Estrogen.swf (animation of steroid hormones entering cell and stimulating transcription)
http://chem.sis.nlm.nih.gov/chemidplus/chemidlite.jsp (basic chem properties and structures)
www.fda.gov
www.niehs.nih.gov
www.hhs.gov

* on the government websites, use the search function to find information on specific plastics additives

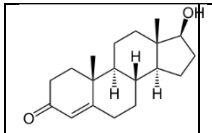
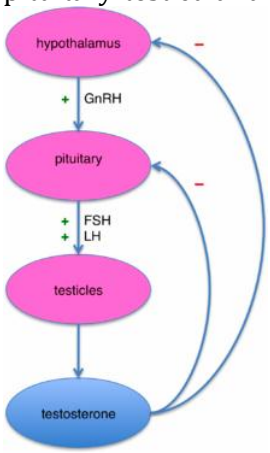
Teacher Resources: Hormone Chart

Hormone	Production Location	Target area	Stimulus for release or production	Effect	Down-regulation mechanism	Method of travel	Other things of interest
Cortisol 	Adrenal gland	Body cells	The release is controlled by the hypothalamus, which responds to stress, or low levels of glucocorticoids in the blood The hypothalamus releases corticotrophin releasing hormone (CRH), which triggers the pituitary to release adrenocorticotrophic (ACTH), which then travels to the adrenal gland via the blood stream and triggers the release of cortisol	Increases blood sugar through gluconeogenesis, suppresses the immune system, and helps in fat, protein and carbohydrate metabolism Ultimately cortisol prevents the release of substances that cause inflammation (a typical immune response)	As cortisol is released into the blood it travels back to the hypothalamus and the pituitary glad to down regulate CRH and ACTH which leads to a drop in production of cortisol (see picture) The hypothalamus-pituitary-adrenal axis 	In the plasma this steroid hormone usually binds to corticosteroid -binding globulin In the cell it typically binds to glucocorticoid receptors	Cortisol levels peak in the early morning and are at their lowest levels about 3 hours after sleep Cortisol levels are irregular in people with clinical depression, psychological stress, or physiological stressors Also known as hydrocortisone
Insulin	The Islets of Langerhans in the pancreas	Body cells	Increases in glucose in the bloodstream indirectly increase calcium levels in the	Lowers blood sugar levels by causing liver, muscle and fat	As sugar levels in the blood decrease, less insulin is released	Since insulin is a protein-like hormone it is water-	Because of its large size, insulin must be injected

			cells. This change then stimulates the pancreas to release stored insulin	cells to take up glucose from the blood Also controls amino acid uptake by cells	from the pancreas	soluble so it doesn't need a transport protein. However, it has receptors in all body cells to help it cross the cell membrane.	directly into fatty tissue.
Leptin	adipose tissue (fatty tissue)	Hypothalamus in the brain	Leptin concentrations are directly proportional to the total amount of fat in the body	Long term appetite suppression by counteracting the effects of two feeding stimulants (neuropeptide Y and anandamide) and stimulating the synthesis of an appetite suppressant.	The less fat cells in an organism, the less total leptin produced.	Protein-like Little is known about how leptin crosses the blood-brain barrier	Anandamide (an appetite stimulant suppressed by leptin) binds to the same receptors as THC note: leptin supplements for obese people have ONLY been shown to work in people in which both genes that code for leptin are mutated
Anti-diuretic hormone (ADH)	Made in the hypothalamus, but released in the pituitary	Kidney	Osmoregulators in the hypothalamus sense increases in the solute concentration of the	ADH decreases the amount of water excreted through urine.	As more water is reabsorbed in the kidney, the concentration of	Protein-like	ADH suppression from alcohol causes

			blood, which then leads to the production and release of ADH	It opens channels in the kidney that allow pure (solute free) water to pass out of the kidney and back into the blood. In other words, if you are dehydrated, ADH will stop you from losing too much water in your urine.	solutes in the blood decrease leading to decreases in release of ADH		dehydration and some of the symptoms of a hangover
Estrogen  estradiol: a common form of estrogen	In women, estrogen is mainly produced in the ovaries In both males and females it is also produced in fat cells	Body cells during female development Bone cells Reproductive organs	Derived from testosterone Production in the gonads is stimulated by the release of Follicle stimulating hormone (FSH from the pituitary gland which is stimulated by the release of gonadotropin releaseing (GnRH) hormone from the hypothalamus	In women, leads to the growth of the reproductive organs, helps regulate the menstrual cycle, and establishes secondary sex characteristics. It also helps to regulate bone growth and bone mass.	As levels of estrogen increase the increased blood concentration prevents the release of GnRH from the hypothalamus. The decrease in GnRH then decreases the production of FSH, which then leads	In the plasma, estrogen is transported by globulin or albumin. It is a steroid, so it can freely cross the cell membrane. Once inside the cell it binds to an estrogen receptor, and	(estro= sexual passion or desire, gen=producer of Used in oral contraceptives

					to decreases in the production of estrogen.	can then cross into the nucleus. There the hormone regulates gene transcription which has the end result of producing the proteins that express the effects.	
Testosterone	Mainly secreted by the testes in males, and the ovaries in	Body cells during male developmen t	Derived from cholesterol Production in the gonads in stimulated	In men, testosterone plays an important role in the development	As levels of testosterone and other androgens increase these levels in the blood	As testosterone is a steroid, it needs a transport	Testosterone is found in both males and females, though it is found in

 a common form of testosterone	females Some is also secreted by the adrenal gland	Reproductive organs	by the release of Follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland which is stimulated by the release of gonadotropin releaseing (GnRH) hormone from the hypothalamus The hypothalamic-pituitary-testicular axis  	of male reproductive tissues like the testes and prostate, as well secondary sex characteristics like body hair and increased muscle mass. In women, testosterone helps to maintain bone density, libido and muscle mass. In general, testosterone promotes protein synthesis and tissue growth for tissues with androgen receptors such as muscles.	prevent the release of GnRH from the hypothalamus, and the decrease in GnRH decreases the production of FSH and LH, which then leads to decreases in the production of androgens such as testosterone.	protein to travel through the blood. It then crosses the membrane alone, and once in the cell attaches to an androgen receptor that carries it to the cell nucleus where it binds to specific DNA sequences and like estrogen, changes the transcriptional activity.	concentrations 7-8 times greater in males. Testoterone is found in almost all vertebrates, even fish. Testosterone can be converted to estrogen by aromatase (CYP19A1) found in the brain liver and fatty tissue. It can also be converted to DHT which is a more potent form of testosterone

Polymer Activity

Pre-Lab: In this lab we will be mixing sodium silicate and ethanol. What do you think will happen when we mix the two? Why?

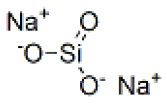
Directions:

1. Observe the properties of ethanol and sodium silicate solution and record them in the table in step 9. Make sure to include viscosity.
2. Measure 20mL of sodium silicate solution in the 100mL graduated cylinder.
3. Pour the sodium silicate solution into the 400mL beaker.
4. Measure 5mL of ethanol in the 10mL graduated cylinder.
5. Add the ethanol to the sodium silicate solution.
6. Stir the solution quickly as the solid begins to form. If the mixture has not formed a solid after 3 minutes of stirring, add 5mL more ethanol.
7. When mixture is solid, remove from beaker using latex gloves.
8. Start molding the mixture into a ball, using paper towels to dry it as you work. Be careful not to mold it too hard as it will crumble.
9. Record the properties of your new polymer in the table below, then clean up your lab station by rinsing out the two graduated cylinders, and scrubbing down the beaker while wearing gloves.

Substance	Properties
Ethanol	
Sodium silicate	
New Polymer	

Follow-up Questions

1. The picture below shows the monomer for the sodium silicate polymer. Draw what the polymer chain might look like. (How do the monomers fit together?)



2. When the sodium silicate and ethanol are mixed together the substance becomes more solid, and much more elastic. What do you think this indicates about the chemical structure of the new substance?

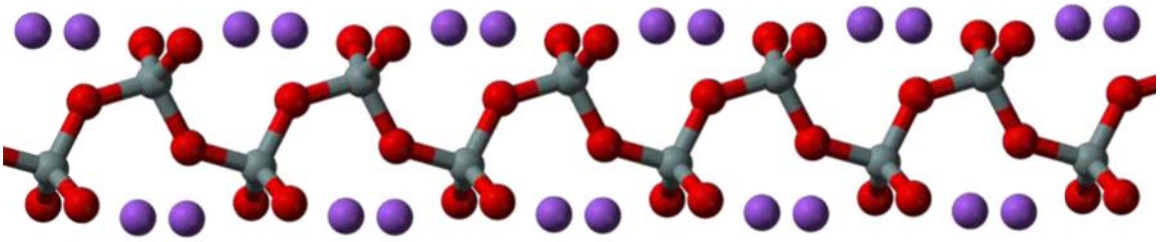
Teacher's Guide

Materials
sodium silicate solution (40%)
ethanol
stir stick
100 mL graduated cylinder
400 mL beaker
10 mL graduated cylinder
latex gloves
paper towels

Sodium silicate can be found online or in chemical supply catalogs.
You can use a disposable stir stick such as a popsicle stick to make clean-up easier.

What's really happening?

Sodium Silicate (Na_2SiO_3) comes in a solution with water. The sodium silicate is often already in polymer form, but when mixed with ethanol, the chain that forms the sodium silicate polymer (see picture below) get linked by the ethanol. The ethanol cross links the separate chains of sodium silicate polymer much the way a chain link fence is linked together. This is what gives the ball its elasticity.



Sodium Silicate Polymer (grey is silica, red is oxygen and purple is sodium)

*Adopted from a demo on the Elmhurst College website (http://www.elmhurst.edu/~chm/vchembook/404silicone.html)

RISE

SCIENTIFIC AMERICAN

Scientific American Magazine - August 26, 2008

Just How Harmful Are Bisphenol-A Plastics?

Patricia Hunt, who helped to bring the issue to light a decade ago, is still trying to sort it all out

By Adam Hinterthuer

On the day Patricia Hunt's career veered into an entirely different field, her graduate students at Case Western Reserve University were grumbling, itching to use some exciting new data in their own experiments, but were told to wait while Hunt (just one last time) checked on her subjects.

Hunt, a geneticist, was exploring why human reproduction is so rife with complications. She had a hunch the chromosomally abnormal eggs that plague human pregnancies were tied to our hormones. A paper outlining the results of Hunt's experiments on the hormone levels of female mice was ready for publication. All she needed was to ensure that her control population, the mice left alone in the study, was normal. Instead Hunt stumbled on a disturbing result—40 percent had egg defects.

Hunt shelved hopes of publication and scrutinized every method and piece of lab equipment used in her experiment. Four months later she finally fingered a suspect.

It was the janitor. In the laboratory. With the floor cleaner.

A single breach in protocol had turned the rodents' safe environs into acutely toxic habitats. A maintenance worker had used an abrasive floor cleaner, instead of the usual mild detergent, to wash out cages and water bottles. The acidic solution scarred the hard, polycarbonate surface of the plastic and enabled a single chemical culprit to leach out—bisphenol-A (BPA).

Hunt's unnerving discovery, in 1998, led her to speak out on the possible human health threats of BPA; she and Frederick vom Saal, a biologist at the University of Missouri–Columbia, have become prominent scientists sounding the alarm. To critics, however, Hunt and vom Saal have been alarmists; they argue that there have been no documented cases of BPA-based plastic harming humans and that fears of the chemical are overblown.

First synthesized in 1891, bisphenol-A came into use as a synthetic estrogen in the 1930s. Later, chemists discovered that, combined with phosgene (used during World War I as a toxic gas) and other compounds, BPA yielded the clear, polycarbonate plastic of shatter-resistant headlights, eyeglass lenses, DVDs and baby bottles.

But during the manufacturing process, not all BPA gets locked into chemical bonds, explains Tim A. Osswald, an expert in polymer engineering at the University of Wisconsin–Madison. That residual BPA can work itself free, especially when the plastic is heated, whether it's a Nalgene bottle in the dishwasher, a food container in the microwave, or a test tube being sterilized in an autoclave.

In recent years dozens of scientists around the globe have linked BPA to myriad health effects in rodents: mammary and prostate cancer, genital defects in males, early onset of puberty in females, obesity and even behavior problems such as attention-deficit hyperactivity disorder.

For her part, the 54-year-old Hunt, now at Washington State University, focuses on aneuploidy, or an abnormal number of chromosomes in eggs that causes birth defects and miscarriages. Last year she co-authored a paper in PLoS Genetics that, she says, makes her original discovery look like "child's play." Hunt exposed pregnant mice to BPA just as the ovaries in their developing female fetuses were producing a lifetime supply of eggs. When the exposed fetuses became adults, 40 percent of their eggs were corrupted, which spelled trouble for their offspring. BPA's effects, it seemed, were not confined to the mouse receiving the dose. "With that one exposure," Hunt says, "we're actually affecting three generations simultaneously."

Although experts debate whether mice make good models for human effects, the crux of the argument over BPA is that experimental results have not been reproduced. A 2004 report from the Harvard Center for Risk Analysis found "no consistent affirmative evidence for low-dose BPA effects." According to I. Glenn Sipes of the University of Arizona, a co-author of that paper, it is this inconsistency that bothers skeptics. "I've never had a problem saying that we can see biological effects in these low-dose studies," he says. "But why are we seeing these studies that can't be repeated?" A onetime result in a rodent model, Sipes argues, cannot be extrapolated to mean negative impacts for human health.

But Hunt counters that there is plenty of corroboration to consider BPA a problem. In response to the Harvard study, she helped to produce a "state of the evidence" paper for *Reproductive Toxicology* in 2007. Along with 36 other researchers, led by vom Saal, the group analyzed hundreds of government-funded studies and found that 90 percent had concluded BPA was a health risk. It was the dozen or so industry-funded studies, vom Saal says, that failed to replicate other BPA research.

More important than these conspiratorial undertones, Hunt says, is one of communication between toxicology (the way skeptics look at BPA) and endocrinology (the way she looks at it). For instance, according to a statement on www.bisphenol-a.org, a Web site created by the American Chemistry Council (which represents dozens of companies engaged in plastics manufacturing), the toxicology of BPA is "well understood," and "BPA exhibits toxic effects only at very high levels of exposure." Current U.S. Food and Drug Administration guidelines, based partly on these findings, set a safe daily exposure to BPA at 50 micrograms per kilogram of body weight.

But according to Hunt, treating BPA like a traditional toxin is dangerous because it "doesn't play by the rules." Standard toxicology states that if a chemical is bad, "then higher doses are worse and an even higher dose is even worse," Hunt explains. But with hormones (and estrogen mimics like BPA), she says, high doses can sometimes "shut down" the body's response, and low doses are enough to exert effects.

Indeed, her lab rodents show BPA effects at just 20 micrograms per kilogram; other labs have found similar thresholds, making them one-half to one-third the FDA levels. These experiments yield bodily concentrations of BPA in ranges of parts per million, but some recent studies have even found that when BPA interacts with hormone receptors on cell membranes, concentrations of one part per *trillion* can stimulate physiological responses.

That means basically any exposure to BPA could have consequences, an alarming conclusion, considering that in 2004 the Centers for Disease Control and Prevention found unmetabolized BPA in the urine of 93 percent of more than 2,500 human subjects. According to the National Toxicology Program of the U.S. Department of Health and Human Services, BPA has also been detected in human blood and breast milk.

With such ubiquitous exposure, one might expect to see numerous problems already afflicting humans. And perhaps this lack of any definitive effects most bothers skeptics. "Why do we have to work so hard to try to replicate and show these low doses really have an effect?" Sipes asks. "Why don't [reactions to BPA] stand out in black and white?"

Hunt is asking the same question. She is now working on a paper about how diet can alter responses to the chemical. It is one of many unstudied facets of the issue that, she says, may be making it difficult for scientists to reproduce their research: "There's a lot of complexity and a lot of things we just don't understand."

While scientists grapple to get a better handle on BPA, the public domain has made up its mind. On April 17 the National Institutes of Health raised concerns about BPA's established "safe" levels. Four days later Health Canada, the Canadian version of the FDA, announced a ban on polycarbonate baby bottles, citing concerns over BPA. The moves rattled the industry, as consumer outcry led stores such as Wal-Mart and CVS to announce they would phase out some polycarbonate products. And Nalgene, a company synonymous with its popular shatter-resistant bottles, decided to pull them from shelves.

The actions may seem premature given the need to solve the mysteries surrounding BPA. But recalling past hazards with mercury and lead in consumer products, Hunt feels caution is justified. "It's not like this has never happened before," she notes. "Now what we have to do is raise awareness and start looking at these products differently—and ask questions about whether they should be making their way into our everyday environment."

Note: This article was originally published with the title, " Safety Dance over Plastic".

RUBRIC- HORMONE PRESENTATIONS

	Below Satisfactory	Max pts	Satisfactory	Max pts	Excellent	Max pts
Where the hormone is produced	incorrect	1	Location is mentioned, but not shown using the organs on the classroom walls	3	Location is mentioned, and shown using the organs on the classroom walls	5
Target cell type and effect	incorrect	10	Some areas unclear in where the target cell is or the final effect of the hormone	25	Addresses in detail both the target cell type, and the effect	30
Regulation of hormone release	incorrect	10	Includes both what initiates the release of the hormone and what stops release of the hormone, but presentation is confusing.	30	Includes both what initiates the release of the hormone and what stops release of the hormone. Pathway is fully and clearly explained using the organs on the classroom walls.	40
How the hormone travels to the inside of the target cell	incorrect	2	Doesn't include type of hormone (protein-like or steroid), or description of membrane crossing is confusing	5	Includes the type of hormone, and how it crosses the cell membrane	10
Overall Clarity of Presentation	Presentation was difficult to follow	5	Some areas of the presentation were unclear, or time limit was exceeded	10	Presentation was concise, within the time limit and included clear explanations.	15
Total						

The Hormone Quiz

See if you can identify the hormone referred to in each statement. You can use each hormone more than once.

1. Lack of this hormone causes super-skinny women to stop having menstrual cycles.

2. This hormone when delivered as a drug is always injected since the protein is too large to pass into the bloodstream by taking it orally.

3. Synthetic versions of this hormone are sometimes illegally used by athletes to increase their muscle mass, and can have side effects such as mood swings in men, and formation of masculine secondary sex characteristics in women.

4. Synthetic versions of this hormone are found in oral contraceptives.

5. An appetite stimulant suppressed by this hormone binds to the same receptors as THC (the potent chemical in marijuana).

6. A synthetic version of this hormone is produced from soy and has anti-inflammatory properties.

7. Levels of this hormone are irregular in people suffering from psychological or physiological stressors.

8. Production of this hormone is suppressed by alcohol, which leads to dehydration and some of the symptoms of a hangover.

9. When taken in large quantities this hormone is converted to estrogen and leads to increases in breast size among males.

The Hormone Quiz-Answer Key

See if you can identify the hormone referred to in each statement. You can use each hormone more than once.

1. Lack of this hormone causes super-skinny women to stop having menstrual cycles.
   ESTROGEN

2. This hormone when delivered as a drug is always injected since the protein is too large to pass into the bloodstream by taking it orally.
   INSULIN

3. Synthetic versions of this hormone are sometimes illegally used by athletes to increase their muscle mass, and can have side effects such as mood swings in men, and formation of masculine secondary sex characteristics in women.
   TESTOSTERONE

4. Synthetic versions of this hormone are found in oral contraceptives.
   ESTROGEN

5. An appetite stimulant suppressed by this hormone binds to the same receptors as THC (the potent chemical in marijuana).
   LEPTIN

6. A synthetic version of this hormone is produced from soy and has anti-inflammatory properties.
   CORTISOL

7. Levels of this hormone are irregular in people suffering from psychological or physiological stressors.
   CORTISOL

8. Production of this hormone is suppressed by alcohol, which leads to dehydration and some of the symptoms of a hangover. **ADH**

9. When taken in large quantities this hormone is converted to estrogen and leads to increases in breast size among males.
   TESTOSTERONE

ARM TWISTING?

Critics of the Bush administration point to headline instances as evidence of its abuse of science, most notably:

- **An attempt to alter an EPA report—in particular, to remove references to a National Academy of Sciences conclusion that humans were contributing to climate change (June 2003).**
- **The dismissal of two scientists from the President's Council on Bioethics after they disagreed with the administration over stem cell research and other issues (February 2004).**
- **The resignation of Susan F. Wood, former director of the Office of Women's Health at the Food and Drug Administration, after the agency overruled its scientific advisory panel and refused to approve over-the-counter sales of an emergency contraceptive known as Plan B, or the morning-after pill (August 2005).**
- **The charges by noted NASA climatologist James E. Hansen that the administration repeatedly tried to stop him from speaking publicly about climate change. Some of the pressure came from George C. Deutsch, a presidential appointee in the NASA public affairs office who later resigned over false academic credentials (January 2006).**

nymity. But "the thing that's disappointing about the amendment is that it doesn't have any enforcement." That is, the department legally cannot violate the provision, but if it does—nothing happens. The amendment, therefore, is likely to have little effect. Nevertheless, Durbin and others see it as an important symbolic step.

The White House did not return calls seeking comment. But the administration did issue a lengthy response when this legislation was introduced. John H. Marburger III, director of the White House Office of Science and Technology Policy, stated then that the administration is "applying the highest scientific standards in decision-making" and that "the accusation of a litmus test that must be met before someone can serve on an advisory panel is preposterous." Marburger himself was appointed to his post despite being "a lifelong Democrat," he said.

With regard to the Miller case, Marburger argued that the National Institute on Drug Abuse had rejected Miller's appointment to the advisory panel on professional grounds, not for political reasons. Such incidents cited by critics represent only a few isolated cases among some 600 scientific committees in the Bush administration, he emphasized. And one of the most important science officials did not experience any meddling: at the annual meeting of the American Association for the Advancement of Science in February, Rita Colwell, who headed the National Science Foundation until 2004, said she had not come under any political pressure during her tenure.

Some of the administration's defenders point out that science and politics have always been strained bedfellows. This administration, they insist, is being unfairly singled out for criticism.

Sheila Jasanoff, a professor of science and technology studies at Harvard University who investigates the use of science in the federal government, disagrees. "Something different is going on in the Bush administration," she claims. Part of the problem is that it attempts to create controversy where none exists. "No matter how good the science is on anything, you can manufacture uncertainty," she says, citing the case of the Environmental Protection Agency giving undue weight to industry studies that question the herbicide atrazine's link to cancer.

Durbin's DHHS funding provision expires in September, at the end of the government's fiscal year. But Durbin has separately introduced legislation that tries to ensure that the federal government will avoid meddling with scientific evidence. It would prohibit censorship of research findings, protect whistle-blowers and keep scientific review out of the hands of the White House.

The legislation's prospects are uncertain. When the amendment was introduced, it attracted 12 cosponsors—all Democrats. Some Republicans have criticized the administration's handling of science, notably Representative Sherwood Boehlert of New York, chair of the House Committee on Science, who urged NASA to stop trying to intimidate James E. Hansen, perhaps the space agency's most famous climatologist. Nevertheless, the administration's critics see the legislation as the beginning of an effort to restore scientific integrity.

Paul Raeburn writes about science, policy and the environment from New York City.

ECOLOGY

Mixing It Up

HARMLESS LEVELS OF CHEMICALS PROVE TOXIC TOGETHER BY DAVID BIELLO

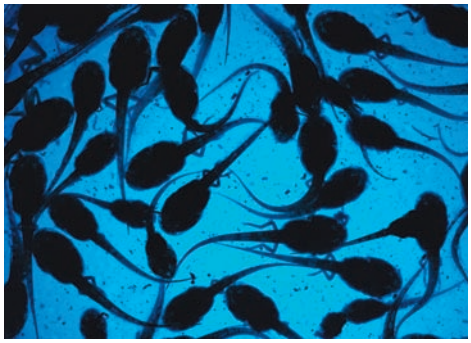
One chemical alone may do no harm in low doses, but in conjunction with a few of its peers, even in doses that are individually safe, it can inflict serious harm. New research in frogs shows that a mixture of nine chemicals found in a seed-corn field in York County, Nebraska, killed a third of exposed tadpoles and lengthened time to metamorphosis by more than two weeks for the survivors.

Biologist Tyrone Hayes and his colleagues at the University of California,

Berkeley, have spent the past four years testing four herbicides, two fungicides and three insecticides commonly used in American cornfields. Individually, the chemicals had little effect on developing tadpoles at low concentrations, such as about 0.1 part per billion. But when Hayes exposed them to all nine at the same low level in the laboratory—the lowest level actually found in the field—the future frogs fell prey to endemic infection. Those that survived ended up smaller than their counterparts raised in clean water—despite taking longer to mature into adults. "In humans, this is like saying, 'The longer you are pregnant, the smaller your baby will be,' which means the womb is no longer a nurturing environment," Hayes notes.

Hayes's study joins a growing body of work showing that chemicals in combination can produce a wide range of effects even at low concentrations. Rick Relyea of the University of Pittsburgh has shown in several studies that tadpoles exposed in their water to low levels of a single pesticide and the smell of a predator will face significantly higher mortality rates. For instance, about 90 percent of bullfrog tadpoles died from exposure to the pesticide carbaryl when the smell of predatory newts was present, whereas no tadpoles perished if exposed to each individually. The pesticide may be inducing a general stress in the tadpole that, when combined with another stressor, becomes deadly, Relyea argues.

It is not just pesticides that show a mixture effect. Phthalates—chemical softeners that make polymers flexible—can interfere with the sexual development of male rats. "We have males treated with phthalates where the testes are under the kidneys or floating around in the abdominal cavity," explains L. Earl Gray, Jr., a biologist at the Environmental Protection Agency and co-discoverer of this deformity, which has been dubbed phthalate syndrome. Gray has also found that various kinds of phthalates in combination either with one another or with certain pesticides and industrial effluents exert ever more powerful effects. For example, two phthalates at concentrations that on their own would not produce much deformity combined to create defective urethras (hypospadias) in 25 percent of exposed rats.



MIXED MESSAGE: Mixtures of pesticides in very low concentrations killed a third of tadpoles and disrupted the development of the survivors.

Besides adding to the issue of endocrine disruption—whether industrial chemicals are mimicking natural hormones—the findings on mixtures pose an incredible challenge for regulators. With tens of thousands of chemicals in regular use worldwide, assessing which combinations might prove harmful is a gargantuan task. "Most of the offices in the agency recognize that we cannot operate via the idea of 'one chemical, one exposure' to an individual anymore. We need to look at broader classes of compounds and how they interact," says Elaine Francis, national program director for the EPA's pesticides and toxics research program. But such testing has a long way to go to reach any kind of regulation, particularly given industry's qualms about the validity of existing research.

Marian Stanley, who chairs the phthalates panel for the American Chemistry Council, notes that at least one study showed that rodents suffering from phthalate malformations could still mate and have litters. "The additivity of phthalates alone are on end points that may not have any biological relevance," she says.

Nevertheless, evidence continues to accumulate that mixture effects are a critical area of study. In its National Water Quality Assessment, the U.S. Geological Survey found that a sampling of the nation's streams contained two or more pesticides 90 percent of the time. "The potential effects of contaminant mixtures on people, aquatic life and fish-eating wildlife are still poorly understood," states hydrologist Robert Gilliom, lead author of the study. "Our results indicate, however, that studies of mixtures should be a high priority."

HUMAN DISRUPTION

Besides affecting amphibians, endocrine disruption—chemical interference with hormonal cascades involved in development—may also be happening in humans. Shanna Swan of the University of Rochester has linked fetal exposure to phthalates and genital changes in 85 baby boys. "We found effects at levels that are seen in a quarter of the U.S. population," Swan says.

But whether the malformations stem from phthalates alone or in combination with other compounds remains unknown, because humans encounter many chemicals in mixture. To help sort out matters, a Johns Hopkins University study will look for the most common chemicals in people. Umbilical cord blood will be tested for a wide array of substances, from pesticides to phthalates to heavy metals, and the overall levels then correlated with the babies' characteristics at birth. Explains the study's leader, Lynn Goldman: "If we can identify some of these mixtures to which people are commonly exposed, then those might be the mixtures to look at more closely."

HEIDI AND HANS-JURGEN KOCH *Minden Pictures*

Phthalates Activity

In groups of 4 or 5, select one study, read the abstract, then selectively read the rest of the study to find the necessary information to answer the questions below.
When you are finished you will share your study with other groups so BE PREPARED to talk about your study.

Studies

-Perinatal Exposure to the Phthalates DEHP, BBP, and DINP, but Not DEP, DMP, or DOTP, Alters Sexual Differentiation of the Male Rat
-Decrease in Anogenital Distance among Male Infants with Prenatal Phthalate Exposure
- The Association between Asthma and Allergic Symptoms in Children and Phthalates in House Dust: A Nested Case–Control Study
- Human Breast Milk Contamination with Phthalates and Alterations of Endogenous Reproductive Hormones in Infants Three Months of Age
- The relationship between environmental exposures to phthalates and DNA damage in human sperm using the neutral comet assay

Background Statistics

The p-value is the probability that the hypothesis is wrong. For instance, if you read a study that gave some mice milk and some mice none and tested weight gain in both groups, the hypothesis is that milk will affect the weight of the mice. The p-value reported is then the likelihood that milk does not affect the weight of the mice. If the p-value is less than 0.05 we often say that it is highly unlikely that the hypothesis is wrong (less than a 5% chance).

The R-value describes the linear relationships among the data points the closer the absolute value of R is to 1, the stronger the linear relationship. An R value of 0 indicates that there is no linear relationship

Questions

1. Record the p-value (and R-value if given) for each hypothesis tested in the study.

p-value (and R-value if given)	Hypothesis tested

2. What are some possible sources of error in the study?

3. What do the author's conclude?

4. What do you take from this study? (your conclusion)

RISE

Follow-Up Questions (Answer after hearing from the other groups)

Do you think there is enough information to warrant regulation of phthalates?

If you were to design new regulations what additional information would you want to know? How could you design a study to address this?

Study	P-values (and R-values if given)	Hypothesis	Possible Sources of Error	Conclusion of the study's authors	Your conclusion

Phthalate Studies Chart

Phthalate Studies Articles

Decrease in Anogenital Distance among Male Infants with Prenatal Phthalate Exposure

*Shanna H. Swan,[1] Katharina M. Main,[2] Fan Liu,[3] Sara L. Stewart,[3] Robin L. Kruse,[3] Antonia M. Calafat,[4] Catherine S. Mao,[5] J. Bruce Redmon,[6] Christine L. Ternand,[7] Shannon Sullivan,[8] J. Lynn Teague,[9] and the Study for Future Families Research Team**

[1]Department of Obstetrics and Gynecology, University of Rochester, Rochester, Minnesota, USA; [2]Department of Growth and Reproduction, University of Copenhagen, Copenhagen, Denmark; [3]Department of Family and Community Medicine, University of Missouri-Columbia, Columbia, Missouri, USA; [4]National Center for Environmental Health, Centers for Disease Control and Prevention, Division of Laboratory Sciences, Atlanta, Georgia, USA; [5]Department of Pediatrics, Division of Endocrinology, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Los Angeles, California, USA; Departments of [6]Pediatrics and [7]Medicine, University of Minnesota Medical School, Minneapolis, Minnesota, USA; [8]Department of Pediatrics, University of Iowa, Iowa City, Iowa, USA; [9]Departments of Surgery (Urology) and Child Health, University of Missouri-Columbia, Columbia, Missouri, USA

Prenatal phthalate exposure impairs testicular function and shortens anogenital distance (AGD) in male rodents. We present data from the first study to examine AGD and other genital measurements in relation to prenatal phthalate exposure in humans. A standardized measure of AGD was obtained in 134 boys 2–36 months of age. AGD was significantly correlated with penile volume (R = 0.27, p = 0.001) and the proportion of boys with incomplete testicular descent (R = 0.20, p = 0.02). We defined the anogenital index (AGI) as AGD divided by weight at examination [AGI = AGD/weight (mm/kg)] and calculated the age-adjusted AGI by regression analysis. We examined nine phthalate monoester metabolites, measured in prenatal urine samples, as predictors of age-adjusted AGI in regression and categorical analyses that included all participants with prenatal urine samples (n = 85). Urinary concentrations of four phthalate metabolites [monoethyl phthalate (MEP), mono-*n*-butyl phthalate (MBP), monobenzyl phthalate (MBzP), and monoisobutyl phthalate (MiBP)] were inversely related to AGI. After adjusting for age at examination, p-values for regression coefficients ranged from 0.007 to 0.097. Comparing boys with prenatal MBP concentration in the highest quartile with those in the lowest quartile, the odds ratio for a shorter than expected AGI was 10.2 (95% confidence interval, 2.5 to 42.2). The corresponding odds ratios for MEP, MBzP, and MiBP were 4.7, 3.8, and 9.1, respectively (all p-values < 0.05). We defined a summary phthalate score to quantify joint exposure to these four phthalate metabolites. The age-adjusted AGI decreased significantly with increasing phthalate score (p-value for slope = 0.009). The associations between male genital development and phthalate exposure seen here are consistent with the phthalate-related syndrome of incomplete virilization that has been reported in prenatally exposed rodents. The median concentrations of phthalate metabolites that are associated with short AGI and incomplete testicular descent are below those found in one-quarter of the female population of the United States, based on a nationwide sample. These data support the hypothesis that prenatal phthalate exposure at environmental levels can adversely affect male reproductive development in humans. *Key words:* anogenital distance, benzylbutyl phthalate, dibutyl phthalate, diethyl phthalate, monobenzyl phthalate, monoethyl phthalate, monoisobutyl phthalate, mono-*n*-butyl phthalate, phthalates, prenatal exposure. *Environ Health Perspect* 113:1056–1061 (2005). doi:10.1289/ehp.8100 available via *http://dx.doi.org/* [Online 27 May 2005]

Diesters of phthalic acid, commonly referred to as phthalates, are widely used in industry and commerce; they are used in personal care products (e.g., makeup, shampoo, and soaps), plastics, paints, and some pesticide formulations. Consistent toxicologic evidence indicates association between several of these phthalate esters and reproductive effects. In particular, dibutyl phthalate (DBP), benzylbutyl phthalate (BzBP), di-2-ethylhexyl phthalate (DEHP), and di-isononyl phthalate have been shown to disrupt reproductive tract development in male rodents in an antiandrogenic manner (Parks et al. 2000). Recent studies have reported significant reductions in anogenital distance (AGD) in Sprague-Dawley rats after prenatal exposure at high doses to BzBP (Nagao et al. 2000; Tyl et al. 2004), DBP (Barlow and Foster 2003; Foster et al. 2000), and DEHP (Gray et al. 2000; Parks et al. 2000).

Despite the growing body of literature on phthalate reproductive toxicity and data demonstrating extensive human exposure (Silva et al. 2004a), few studies have examined the effects of these chemicals on human reproductive development. Colón et al. (2000) reported elevated levels of several phthalates [including diethyl phthalate (DEP), DBP, and DEHP] in serum samples from young girls with premature breast development. However, the timing of exposure was unknown and high exposure levels may have reflected phthalate contamination of serum samples (McKee and Toxicology Research Task Group 2004). Until recently, the only study of humans to evaluate phthalate exposure and male reproductive toxicity measured phthalate diesters in semen. As with the Colón et al. study, contamination from diesters in laboratory equipment could not be excluded (Murature et al. 1987).

More recent studies have examined phthalate monoester metabolites in urine. Because urinary metabolites are not likely to be present as the result of contamination, these studies avoid this potential source of measurement error. Duty et al. (2003a) reported dose–response relationships between tertiles of monobutyl phthalate and sperm motility and sperm concentration, and between tertiles of monobenzyl phthalate (MBzP) and sperm concentration. They also reported inverse dose–response relationships between monoethyl phthalate (MEP) and sperm DNA damage

Address correspondence to S.H. Swan, University of Rochester, Department of Obstetrics and Gynecology, School of Medicine and Dentistry, 601 Elmwood Ave., Box 668, Rochester, NY 14642-8668 USA. Telephone: (585) 273-3521. Fax: (585) 275-7366. E-mail: shanna_swan@urmc.rochester.edu

*The Study for Future Families Research Team included, from the University of Missouri-Columbia: E.Z. Drobnis, B.S. Carter, D. Kelly, and T.M. Simmons. Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center: C. Wang, L. Lumbreras, S. Villanueva, M. Diaz-Romero, M.B. Lomeli, and E. Otero-Salazar. Cedars-Sinai Medical Center: C. Hobel and B. Brock. University of Minnesota: C. Kwong and A. Muehlen. University of Iowa: A. Sparks, A. Wolf, J. Whitham, M. Hatterman-Zogg, and M. Maifeld.

We thank the health care providers and study participants at University Physicians Clinic (Columbia, MO), Fairview Riverside Women's Clinic (Minneapolis, MN), Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center (Los Angeles, CA), Cedars-Sinai Medical Center (Los Angeles, CA), and University of Iowa Hospitals and Clinics (Iowa City, IA). We also thank M. Silva, J. Reidy, E. Samandar, and J. Preau for phthalate analyses and E. Gray, P. Foster, and D. Barr for their guidance.

This work was supported by grants from the U.S. Environmental Protection Agency and the National Institutes of Health (R01-ES09916 to the University of Missouri, MO1-RR00400 to the University of Minnesota, MO1-RR0425 to Harbor-UCLA Medical Center) and by grant 18018278 from the State of Iowa to the University of Iowa.

The authors declare they have no competing financial interests.

Received 7 March 2005; accepted 25 May 2005.

measured using the neutral single-cell gel electrophoresis (comet) assay (Duty et al. 2003b). In this population of men attending an infertility clinic, increased urinary concentration of MBzP was also associated with decreased follicle stimulating hormone, whereas increases in monobutyl phthalate were marginally associated with increased inhibin-B (Duty et al. 2005).

Newborn male rodents have no scrotum, and the external genitalia are undeveloped; only a genital tubercle is apparent for both sexes. The distance from the anus to the insertion of this tubercle, the AGD, is androgen dependent and about twice as long in males as in females. The AGD has been shown to be a sensitive measure of prenatal antiandrogen exposure (Rhees et al. 1997). Recently, Salazar-Martinez et al. (2004) studied AGD in 45 male and 42 female infants. They measured the distance from the anus to the base of the scrotum in males and from the anus to the base of the genitals (the fourchette) in females. By these measures, AGD was sexually dimorphic and about twice as long in males as in females. No other studies have examined AGD among human males, although two other studies have evaluated AGD in female infants (Callegari et al. 1987; Phillip et al. 1996).

Materials and Methods

Study participants. Women included in our study were originally recruited into the first phase of the Study for Future Families (SFFI), a multicenter pregnancy cohort study, at prenatal clinics in Los Angeles, California (Harbor-UCLA and Cedars-Sinai), Minneapolis, Minnesota (University of Minnesota Health Center), and Columbia, Missouri (University Physicians), from September 1999 through August 2002. Data collection is still ongoing in Iowa, where a center was added late in SFFI, so Iowa participants are not included in this analysis. Methods are described in detail elsewhere (Swan et al. 2003). Briefly, couples whose pregnancy was not medically assisted were eligible unless the woman or her partner was < 18 years of age, either partner did not read and speak Spanish or English, or the father was unavailable or unknown. All participants completed a questionnaire, most gave blood samples, and after urine collection was added midway through the study, most also gave a urine sample.

Eighty-five percent of SFFI participants agreed to be recontacted, and we invited these mothers to take part in our follow-up study. The family was eligible for the follow-up study (SFFII) if the pregnancy ended in a live birth, the baby was 2–36 months of age, and the mother lived within 50 mi of the clinic and could attend at least one study visit. Here we report on results from the first study visit only. Human subject committees at all participating institutions approved SFFI and SFFII, and all participants signed informed consents for each study.

Physical examination. After standard anthropometric measurements (height, weight, head circumference, and skin-fold thickness) were obtained, a detailed examination of the breast and genitals was conducted under the supervision of pediatric physicians who were trained in its administration. Every attempt was made to standardize the examination, which was developed specifically for this study. These methods included training sessions before and during the study and the use of standardized equipment. Neither the pediatric physicians nor the support staff had any knowledge of the mother's phthalate concentrations.

Boys' genital examinations included a description of the testes and scrotum, location and size of each testicle, and measurement of the penis. The placement of each testicle was initially coded in six categories; in the present analysis, boys are dichotomized into those with normal testicular descent (placement of both testes coded as normal or normal retractile) or with incomplete testicular descent (all other cases). The scrotum was categorized as distinct from surrounding tissue or not, and by size (small or not). Penile width and (stretched) length were recorded, and penile volume [proportional to (penile width/2)^2 × penile length] was calculated. We recorded the AGD, measured from the center of the anus to the anterior base of the penis. We also recorded the anoscrotal distance (ASD), measured from the center of the anus to the posterior base of the scrotum. This latter measurement was used by Salazar-Martinez et al. (2004), who refer to it as AGD.

Phthalate metabolite analysis. Urinary phthalate metabolite analyses were carried out by the Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention (CDC), which had no access to participant data. The analytical approach for the analysis of urinary phthalate metabolites (Silva et al. 2004b) is a modification of previously published methods (Silva et al. 2003). The analysis involves the enzymatic deconjugation of the phthalate metabolites from their glucuronidated form, automated on-line solid-phase extraction, separation with high-performance liquid chromatography, and detection by isotope-dilution tandem mass spectrometry. This high-throughput method allows for the simultaneous quantification in human urine of the nine phthalate metabolites reported in this work. Limits of detection (LOD) are in the low nanogram per milliliter range. Isotopically labeled internal standards were used along with conjugated internal standards to increase precision and accuracy of the measurements. The method is accurate (spiked recoveries are near 100%), and precise with between-day relative standard deviations of < 10%. Quality control (QC) samples and laboratory blanks were analyzed along with unknown samples to monitor performance of the method. The metabolite concentrations reported here are from 85 prenatal maternal urine samples of a total of 214 that also included postnatal maternal and baby samples from the same mothers and their children. The 214 samples were analyzed for phthalate metabolites in six batches, none of which had to be re-extracted for QC failures. Of the 214 samples, seven were re-extracted using < 1 mL of urine because concentrations of MEP calculated using 1 mL were above the linear range of the method.

Statistical analysis. After examining descriptive and summary statistics for all study variables, we explored models for AGD. We fit several alternative measures of body size (weight, height, and body mass index) and both additive and multiplicative functions of these. We defined the anogenital index [AGI = AGD/weight (mm/kg)] as a weight-normalized index of AGD.

AGD and AGI were modeled as both linear and quadratic functions of age. For babies born at < 38 weeks, age at examination in the first year was calculated from the estimated date of conception instead of the birth date. Once the best fitting model was identified, we plotted the expected AGI and its 25th and 75th percentiles as a function of age. We categorized boys in two ways: We dichotomized boys into those with AGI smaller than or at least as large as expected, and we used the difference between observed and expected AGI to define three groups of boys, short (AGI < 25th percentile for age), intermediate (25th percentile ≤ AGI < 75th percentile), and long (AGI ≥ 75th percentile for age) AGI. We also calculated the proportion of boys in these three groups with normal testicular descent (both testes normal or normal retractile) and normal scrotal (scrotum of normal size and distinct from surrounding tissue). We calculated the correlations between AGD and AGI and penile volume, testicular placement and scrotal parameters (size and distinctness from surrounding tissue). Our decision to use AGI as the measure of genital development was made, and cut points for categorical analyses of outcomes were selected, before obtaining phthalate metabolite values.

We used general linear models to explore the relationships between phthalate metabolite concentration (unadjusted for urine concentration) and genital parameters. Most metabolite concentrations were above the LOD; those below the LOD were assigned the value LOD divided by the square root of 2, which has been recommended when the data are not highly skewed, as was the case here (Hornung and

Reed 1990). Metabolite concentrations were logarithmically transformed to normalize distributions. We examined several potentially confounding factors including mother's ethnicity and smoking status, time of day and season in which the urine sample was collected, gestational age at sample collection, and baby's weight at examination.

We also categorized metabolite concentrations into low (< 25th percentile), intermediate (between the 25th and 75th percentiles), and high (≥ 75th percentile) categories and examined the odds ratio (OR) for smaller than expected AGI for babies with high compared with low exposure, and medium compared with low. On the basis of these regression and categorical analyses, we identified the phthalate metabolites most strongly associated with AGI. We refer to these as AGI-associated phthalates.

Because phthalate metabolite concentrations are highly correlated, and because our limited sample size prohibited us from examining multiway interactions, we constructed a summary phthalate score to examine the effect of joint exposure to more than one AGI-associated phthalate. For this purpose, we used quartiles of metabolite concentration; values in the lowest quartile did not contribute to the sum, whereas higher values increased the sum one unit per quartile. We divided this sum into three categories: low (0–1, reflecting little or no exposure to AGI-associated phthalates), intermediate (2–10), and high (11–12, reflecting high exposure to all, or almost all, AGI-associated phthalates). We examined the magnitude of the residual (observed – expected) AGI as a function of this summary phthalate score.

Results

The population for the present analysis was identified from families recruited in California, Minnesota, or Missouri for whom data entry was complete by 17 December 2004, the cutoff date for the present analysis. At that time, 654 participants from these three centers had completed SFFI and given permission to be recontacted. Of these, 477 (72.9%) were eligible for SFFII and 346 (72.5%) participated (Table 1). SFFII participants were demographically similar to nonparticipants except that nonparticipants were more likely to be Hispanic because of a lower eligibility rate (60%) in CA, where most participants were Hispanic. Of the 172 boys born to these mothers, we excluded 5 boys in twin births, 10 boys with incomplete data, and 23 boys for whom AGD was not recorded [two whose mothers declined the genital exam, with the remainder older boys (mean age, 19.6 months), for whom the study examiner felt the measurement was not reliable, usually because of the boys' activity level]. The remaining 134 boys comprise the sample used for the analysis of AGD and other genital measurements. Among the 134 boys for whom we have genital measurements, no frank genital malformations or disease were detected, and no parameters appeared grossly abnormal. The mean age at first examination was 15.9 months, and mean weight was 10.5 kg (Table 2). Mean (± SD) AGD was 70.3 ± 11.0 mm, with a distribution that was well approximated by a normal curve. Overall, 86.6% of boys had both testes classified as normal or normal-retractile.

A prenatal urine sample was assayed for phthalate metabolites for mothers of 85 of these boys. These mother–son pairs comprise the data set for the analysis of AGD and phthalate metabolite concentration. Because urine collection began midway through SFFI, mothers with a stored urine sample were recruited later in the study, and their sons tended to be younger at examination (mean age, 12.6 months; interquartile range, 5–16 months). Summary statistics for all boys included in the analysis of physical measurements, and the subset of boys for whom mothers' prenatal phthalate concentrations were also available are shown separately in Table 2.

All phthalate metabolites tested were above the LOD in > 49% of women, and most tested were above the LOD in > 90% of the samples (Table 3). Concentrations spanned four orders of magnitude, from below the LOD (estimated value = 0.71 ng/mL) to 13,700 ng/mL for MEP. Means ranged from 2.68 for mono-3-carboxypropyl phthalate (MCPP) to 629.8 for MEP. Three of the four AGI-associated metabolites (other than MEP) were significantly correlated ($p < 0.005$).

Regression analyses. We initially modeled AGD as a linear function of age and weight, but this model fit poorly (adjusted $R^2 = 0.22$). We found that using AGI (AGD/weight) as a function of age provided the best fit, as has been shown in rodent models (Vandenbergh and Huggett 1995). The best-fitting model for AGI includes linear and quadratic terms for age and is given by AGI = 10.8835 – 0.3798 (age) + 0.0068 (age^2) (adjusted $R^2 = 0.61$). Using this model, we calculated mean AGI and its 5th, 25th, 75th, and 95th percentiles (Figure 1).

We then examined models that included individual phthalate metabolites. Other than age and age squared, no covariates altered regression coefficients for the phthalate metabolites by > 15%, and none were included in final models. All regression coefficients for individual metabolites (logarithmically transformed to normalize distributions) were negative (Table 4). MEP, mono-*n*-butyl phthalate (MBP), MBzP, and monoisobutyl phthalate (MiBP) were (inversely) related to AGI; *p*-values for regression coefficients were between 0.007 and 0.097. We also measured three metabolites of DEHP. Although the hydrolytic monoester metabolite mono-2-ethylhexyl phthalate (MEHP) was unrelated to AGI [regression coefficient = –0.05; 95% confidence interval (CI), –0.53 to 0.43], regression

Table 1. Participants included in present analysis.

	No.	Percent potential participants	Percent male babies
All pregnancy outcomes (CA, MN, and MO)			
Potential participants[a]	654	100	—
Eligible for SFFII	477	72.9	—
SFFII participant	346	72.5	—
Male babies only (CA, MN, and MO)			
SFFII participant	172	—	100
With AGD, age, and weight[b]	134	—	78
Prenatal urine sample[c]	85	—	49

[a]A potential participant is an SFFI participant from CA, MO, or MN who gave permission to be recontacted for future studies and for whom all study data were entered by 17 December 2004. [b]Boys in twin births and boys with missing data or AGD measurements considered unreliable by pediatricians excluded. [c]Urine collection began midway through SFFI.

Table 2. Characteristics of boys with complete physical examination.

Characteristic	Mean ± SD	Percentile 25th	50th	75th
All boys (*n* = 134)				
Age (months)	15.9 ± 8.6	11.0	15.0	23.0
Height (cm)	79.1 ± 10.6	72.6	80.0	87.2
Weight (kg)	10.5 ± 2.7	8.7	10.7	12.3
AGD (mm)	70.3 ± 11.0	63.9	70.3	76.6
AGI (mm/kg)	7.1 ± 1.9	5.8	6.7	7.8
ASD (mm)	37.4 ± 7.5	31.2	36.8	43.4
Boys whose mother's prenatal urine was assayed for phthalate metabolites (*n* = 85)				
Age (months)	12.6 ± 6.9	5.0	14.0	16.0
Height (cm)	75.6 ± 9.5	66.5	77.6	82.0
Weight (kg)	9.7 ± 2.4	8.4	10.0	11.1
AGD (mm)	68.0 ± 9.7	61.7	66.7	74.4
AGI (mm/kg)	7.4 ± 1.8	6.1	7.0	8.2
ASD (mm)	35.9 ± 7.1	30.4	35.6	41.4

coefficients for the oxidative monoester metabolites of DEHP, mono-2-ethyl-5-oxohexyl phthalate (MEOHP), and mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP) were of a magnitude comparable with those for MEP and MBzP (p-values = 0.114 and 0.145 for MEOHP and MEHHP, respectively). AGI appeared to be independent of the concentrations of monomethyl phthalate (MMP) and MCPP, metabolites of dimethyl phthalate and di-*n*-octyl phthalate, respectively.

Categorical analyses. The 25 boys with AGI below the 25th percentile for age were classified as having a short AGI. This group had an AGI that was, on average, 18.3% (range, 10–32%) shorter than expected based on the final regression model. Boys with AGI ≥ 75th percentile of expected were classified as having a long AGI, and boys with AGI between the 25th and 75th percentile of expected were considered intermediate. Boys' weight and age did not differ appreciably among these groups.

Table 5 shows mean and median values for the AGI-associated metabolites for boys in the short, intermediate, and long categories of AGI. We calculated the ORs for short AGI for each monoester metabolite (Table 6). For high compared with low concentration of MBP, the OR for a short AGI was 10.2 (95% CI, 2.5 to 42.2), whereas for medium concentration compared with low the OR was 3.8 (95% CI, 1.2 to 12.3). The corresponding ORs for high compared with low concentration of MEP, MBzP, and MiBP were 4.7, 3.8, and 9.1, respectively (all p-values < 0.05).

Other genital parameters. Degree of testicular descent was associated with AGD (R = 0.20, p = 0.02). The proportions of boys with one or both testicles incompletely descended were 20.0, 9.5, and 5.9% for boys classified as having short, intermediate, and long AGI (p-value for short AGI compared with all other boys < 0.001). The proportion of boys with a scrotum categorized as small and/or "not distinct from surrounding tissue" was also elevated for boys with short AGI (p < 0.001). AGD was significantly associated with penile volume (R = 0.27, p = 0.001), and penile volume divided by weight was correlated with AGI (R = 0.43, p = 0.001). Testicular volume, which was measured by orchidometer, is not shown here because participating physicians considered the measurement to be unreliable—a decision made before analyses of phthalate exposure.

ASD was, on average, 47% as long as AGD, and these two measurements were correlated (R = 0.47, p < 0.0001). However, the model predicting ASD as a function of baby's age and weight fit poorly (adjusted R^2 = 0.10). The fit for the model using ASD/weight as a function of age and age squared was better (adjusted R^2 = 0.47) but did not fit as well as the model using AGI (R^2 = 0.61). ASD/weight was associated with MEP concentration (regression coefficient = –0.429; 95% CI, –0.722 to –0.137). For the other phthalate metabolites, regression coefficients were less significant (all p-values between 0.11 and 0.97).

Summary phthalate score. We used the summary phthalate score as defined in "Materials and Methods" to study the effect of joint exposure to more than one AGI-associated phthalate. The summary phthalate score was directly related to the proportion of boys with short AGI (p = 0.001). Of the 10 boys whose phthalate scores were high (score = 11–12), all but one had a short AGI. Conversely, of the 11 boys whose scores were low (score = 0 or 1), only one had a short AGI. The ORs for having a short AGI for high summary phthalate score compared with low (OR = 90.0; 95% CI, 4.88 to 1,659), and high compared with medium (29.4; 95% CI, 3.4 to 251) were large and significant, although the confidence intervals were very wide. These data are shown graphically in Figure 1.

Discussion

In the recent National Health and Nutrition Examination Survey (NHANES 1999–2000), most of the general population in the United States had measurable exposure to multiple phthalates (CDC 2003; Silva et al. 2004a). The samples in the present study and in NHANES were both analyzed using comparable methods and standards by the same laboratory, although the specific metabolites that were measured in the two studies differed somewhat. We compared the medians and 75th percentiles of the AGI-associated phthalate metabolite concentrations among two groups of mothers in our study (those whose boys fell in the short AGI group and all others) with those of females in the NHANES sample (Table 7). In the analysis of the NHANES samples, monobutyl phthalate includes both MBP and MiBP, which were measured separately in our study. Metabolite concentrations for mothers of boys with short AGI were consistently higher than those of other mothers. Compared with women in the NHANES sample, metabolite concentrations for our population were somewhat lower. However, our population cannot be directly compared with

Table 3. Percentiles of phthalate monoester metabolites.

Monoester metabolite	Percentile (ng/mL) 25th	50th	75th	Percent > LOD[a]
Phthalate monoester metabolite				
MBP	7.2	13.5	30.9	96.5
MBzP	3.5	8.3	23.5	94.1
MCPP	0.7	2.1	3.6	69.4
MEP	53.3	128.4	436.9	97.6
MiBP	0.7	2.5	5.1	74.1
MMP	0.7	0.7	3.2	49.4
Metabolites of DEHP				
MEHHP	6.0	11.4	20.1	97.6
MEHP	1.3	3.3	9.0	77.6
MEOHP	5.1	11.1	19.0	94.1

[a]LOD for all metabolites was between 0.95 and 1.07 ng/mL.

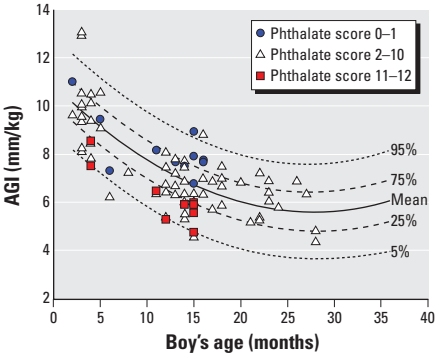


Figure 1. Mean AGI (mm/kg) in relation to boys' age at examination (months).

Table 4. Regression analyses of AGI on $\log_{10}$ monoester metabolite concentration, controlling for age and age squared.

Monoester metabolite	$\log_{10}$ monoester metabolite concentration Coefficient (SE)	p-Value (95% CI)
MBP	–0.592 (0.269)	0.031 (–1.126 to –0.057)
MBzP	–0.390 (0.232)	0.097 (–0.851 to 0.072)
MCPP	–0.264 (0.356)	0.461 (–0.973 to 0.445)
MEHHP	–0.398 (0.270)	0.145 (–0.935 to 0.140)
MEHP	–0.051 (0.241)	0.833 (–0.530 to 0.428)
MEOHP	–0.412 (0.258)	0.114 (–0.925 to 0.101)
MEP	–0.400 (0.164)	0.017 (–0.726 to –0.074)
MiBP	–0.765 (0.274)	0.007 (–1.309 to –0.220)
MMP	–0.283 (0.323)	0.383 (–0.924 to 0.359)
Phthalate score[a]	–0.0951 (0.035)	0.009 (–0.165 to –0.025)

[a]Phthalate score measures joint exposure to MBP, MBzP, MEP, and MiBP; see "Statistical analysis."

NHANES: the proportion of pregnant women in the NHANES sample is unknown, and age distributions differ. Nonetheless, these data demonstrate that the four AGI-associated phthalate metabolites are prevalent in the U.S. female population, and levels were not unusually high among mothers whose sons had a short AGI.

Although not identical, AGD in pups is most similar to AGD as we defined it in this study. In rodents, AGD has been shown to be one of the most sensitive end points for phthalates such as DBP (Mylchreest et al. 2000) and other antiandrogens such as flutamide (Barlow and Foster 2003; McIntyre et al. 2001) and finasteride (Bowman et al. 2003). It is difficult to compare the dose to humans from low-level, ongoing, environmental exposure with that delivered to rodents experimentally in a narrow window of gestation. Nonetheless, it is likely that the doses to which our participants were exposed are lower than those used in toxicologic settings, suggesting that humans may be more sensitive to prenatal phthalate exposure than rodents. This greater sensitivity in humans has been observed for other toxicants. For example, humans are more sensitive to trenbolone by an order of magnitude (Neumann 1976). This greater sensitivity is thought to be a result of rodents' higher metabolic rate and more rapid inactivation of toxicants, both of which have been shown to be inversely related to body size (White and Seymour 2005).

In light of the toxicologic literature for MBP, MBzP, and MiBP (Ema et al. 2003; Foster et al. 1980, 1981; Gray et al. 2000; Nakahara et al. 2003), our data suggest that the end points affected by these phthalates are quite consistent across species. A boy with short AGI has, on average, an AGI that is 18% shorter than expected based on his age and weight as well as an increased likelihood of testicular maldescent, small and indistinct scrotum, and smaller penile size. These changes in AGD and testicular descent are consistent with those reported in rodent studies after high-dose phthalate exposure (Ema et al. 2003; Gray et al. 2000; Mylchreest et al. 2000). The lack of association for MCPP and MMP, which have not been widely studied, is not inconsistent with the toxicologic literature.

With respect to DEP and its metabolite MEP, we note that there are three other human studies suggesting reproductive toxicity (Colón et al. 2000; Duty et al. 2003b; Main KM, unpublished data). It is therefore uncertain whether the absence of data in rodents showing reproductive toxicity is the result of failure to detect it, unmeasured confounding in human studies, or interspecies differences in response to these compounds.

DEHP has been shown to shorten AGD (Gray et al. 2000) and reduce testosterone (Parks et al. 2000). Although MEHP was not associated with AGD in our data, the associations for the oxidative metabolites of DEHP (MEOHP and MEHHP) were of comparable magnitude with those for metabolites of DBP and BzBP, although not statistically significant. Thus, it is unclear whether MEOHP and MEHHP are (inversely) associated with AGI, although associations are of borderline statistical significance because of our sample size, or whether human and rodent responses to this phthalate and its metabolites differ.

Masculinization of external male genitalia, represented by longer AGD, is controlled by dihydrotestosterone (Clark et al. 1990). Ema and Miyawaki (2001) demonstrated that this metabolite of testosterone is markedly decreased by prenatal administration of MBP, suggesting that MBP acts as an antiandrogen. AGD in male rodents is associated with other adverse developmental effects (Foster and McIntyre 2002) and some phthalate-induced changes have been shown to be permanent. For example, Barlow et al. (2004) report that prenatal exposure to 500 mg/kg/day DBP resulted in permanently decreased AGD and testicular dysgenesis. They also report that *in utero* DBP exposure induced proliferative Leydig cell lesions. Follow-up of exposed children until adulthood will be required to determine whether long-term effects, including testicular dysgenesis, are seen in humans after prenatal phthalate exposure.

Several recent studies of the variability of phthalate monoester concentration in human samples suggest that phthalate concentration in humans is fairly stable, perhaps reflecting habitual use of phthalate-containing household and consumer products (Colón et al. 2000; Hauser et al. 2004; Hoppin et al. 2002). These studies lend support to the use of a single sample for exposure assessment. We obtained only a single prenatal urine sample from each woman, and most samples were obtained quite late in pregnancy (mean = 28.3 weeks). Therefore, the measured phthalate metabolite levels may not reflect exposure during the most sensitive developmental window, resulting in some degree of exposure misclassification. However, unless this misclassification varied systematically with outcome, such errors would bias the effect estimate toward the null. In fact, the categorical analysis, which should be less sensitive to such misclassification, showed

Table 5. Mean (median) phthalate monoester metabolite levels by AGI category.

Monoester metabolite	AGI category [mean (median; ng/mL)]		
	Long[a] (n = 17)	Intermediate[b] (n = 43)	Short[c] (n = 25)
MBP	13.1 (11.5)	22.2 (13.1)	38.7 (24.5)
MBzP	10.6 (6.6)	15.1 (7.7)	25.8 (16.1)
MEP	124 (47.1)	592 (112)	1,076 (225)
MiBP	2.3 (1.5)	3.3 (2.1)	7.7 (4.8)

[a]Long, AGI ≥ 75th percentile of expected AGI. [b]Intermediate, 25th percentile ≤ AGI < 75th percentile of expected AGI. [c]Short, AGI < 25th percentile of expected AGI.

Table 6. OR (95% CI) for AGI less than expected from regression model, by monoester metabolite level.

Monoester metabolite	Level (percentile)	AGI < expected	AGI ≥ expected	OR (95% CI)
MBP	Low	5	15	Referent
	Medium	24	19	3.8 (1.2 to 12.3)
	High	17	5	10.2 (2.5 to 42.2)
MBzP	Low	6	13	Referent
	Medium	26	18	3.1 (1.002 to 9.8)
	High	14	8	3.8 (1.03 to 13.9)
MEP	Low	7	14	Referent
	Medium	25	19	2.6 (0.9 to 7.8)
	High	14	6	4.7 (1.2 to 17.4)
MiBP	Low	6	16	Referent
	Medium	23	18	3.4 (1.1 to 10.5)
	High	17	5	9.1 (2.3 to 35.7)

Low, < 25th percentile; medium, ≥ 25th and < 75th percentile; high, ≥ 75th percentile.

Table 7. Concentrations of four phthalate metabolites in three groups of women (ng/mL).

Monoester metabolite	Percentile	This study		NHANES[a]
		Short AGI	Others	
MBP	50th	24.5	12.1	30.0
	75th	44.8	28.0	59.5
MBzP	50th	16.1	7.2	16.0
	75th	27.5	17.8	35.8
MEP	50th	225	90.4	174
	75th	551	281	425
MiBP	50th	4.8	2.1	—[b]
	75th	12.1	4.3	—[b]

[a]Females only (CDC 2003). [b]MBP in the NHANES analysis includes both MBP and MiBP; in this study these metabolites were measured separately.

stronger associations than did the continuous analysis.

Our analysis is based on a single measure of AGD, and the reliability of this measurement in humans has not been established. During two training sessions, three study physicians each measured AGD in four male infants (mean age, 8.1 months). The mean AGD for these measurements was 58.6 mm, SD was (within infant) 4.2 mm, and coefficient of variation of 7.2%, suggesting that AGD can be measured reliably. Use of this measurement in larger studies in a range of diverse populations, with many more such training sessions, will be needed to obtain normative data.

Although it might have been ideal to examine babies shortly after birth, the timing of grant funding did not allow this. Babies were born to SFFI mothers as early as January 2000, and the first baby visits did not occur until April 2002. To maximize the number of children participating, we allowed recruitment over a range of ages. On the other hand, because the use of AGD in humans is new, the optimal timing for this measurement is not known. Our data suggest that measurements are reliable and informative in young children at least until 18 months, when AGD becomes more difficult to obtain reliably. Its value in adolescents and adults has yet to be determined.

We note that phthalate metabolite levels were highly correlated, and most women were exposed to all metabolites at detectable levels. Gray et al. (2000) suggested that risk assessments for phthalate-induced reproductive toxicity should consider phthalates as a group and include exposures from multiple sources. The score we use reflects joint exposure to the four AGI-associated phthalates, and our results suggest that joint exposure may convey greater than additive risk, but larger sample sizes are needed to confirm this.

Gray and Foster (2003) refer to a "phthalate syndrome" characterized by testicular, epididymal, and gubernacular cord agenesis as well as decreased AGD, and stress the importance of evaluating all components of a syndrome so that affected animals are not misidentified. It has recently been suggested (Fisher 2004) that this "phthalate syndrome" shares many features with the human testicular dysgenesis syndrome proposed by Skakkebaek et al. (2001) to follow chemically induced disruption of embryonic programming and gonadal development during fetal life. The present findings, though based on small numbers, provide the first data in humans linking measured levels of prenatal phthalates to outcomes that are consistent with this proposed syndrome.

This is the first study to look at subtle patterns of genital morphology in humans in relation to any prenatal exposure. It was motivated by toxicologic studies showing that genital morphology is altered by antiandrogens, including some phthalates. We report that AGD, the most sensitive marker of antiandrogen action in toxicologic studies, is shortened and testicular descent impaired in boys whose mothers had elevated prenatal phthalate exposure. These changes in male infants, associated with prenatal exposure to some of the same phthalate metabolites that cause similar alterations in male rodents, suggest that commonly used phthalates may undervirilize humans as well as rodents.

References

Barlow NJ, Foster PM. 2003. Pathogenesis of male reproductive tract lesions from gestation through adulthood following *in utero* exposure to di(*n*-butyl) phthalate. Toxicol Pathol 31:397–410.

Barlow NJ, McIntyre BS, Foster PM. 2004. Male reproductive tract lesions at 6, 12, and 18 months of age following *in utero* exposure to di(*n*-butyl) phthalate. Toxicol Pathol 32:79–90.

Bowman CJ, Barlow NJ, Turner KJ, Wallace DG, Foster PM. 2003. Effects of *in utero* exposure to finasteride on androgen-dependent reproductive development in the male rat. Toxicol Sci 74:393–406.

Callegari C, Everett S, Ross M, Brasel JA. 1987. Anogenital ratio: measure of fetal virilization in premature and full-term newborn infants. J Pediatr 111:240–243.

CDC. 2003. Second National Report on Human Exposure to Environmental Chemicals. Atlanta, GA:Centers for Disease Control and Prevention, National Center for Environmental Health, Division of Laboratory Sciences.

Clark RL, Antonello JM, Grossman SJ, Wise LD, Anderson C, Bagdon WJ, et al. 1990. External genitalia abnormalities in male rats exposed in utero to finasteride, a 5 alpha-reductase inhibitor. Teratology 42:91–100.

Colón I, Caro D, Bourdony CJ, Rosario O. 2000. Identification of phthalate esters in the serum of young Puerto Rican girls with premature breast development. Environ Health Perspect 108:895–900.

Duty SM, Calafat AM, Silva MJ, Ryan L, Hauser R. 2005. Phthalate exposure and reproductive hormones in adult men. Hum Reprod 20:604–610.

Duty SM, Silva MJ, Barr DB, Brock JW, Ryan L, Chen Z, et al. 2003a. Phthalate exposure and human semen parameters. Epidemiology 14:269–277.

Duty SM, Singh NP, Silva MJ, Barr DB, Brock JW, Ryan L, et al. 2003b. The relationship between environmental exposures to phthalates and DNA damage in human sperm using the neutral comet assay. Environ Health Perspect 111:1164–1169.

Ema M, Miyawaki E. 2001. Adverse effects on development of the reproductive system in male offspring of rats given monobutyl phthalate, a metabolite of dibutyl phthalate, during late pregnancy. Reprod Toxicol 15:189–194.

Ema M, Miyawaki E, Hirose A, Kamata E. 2003. Decreased anogenital distance and increased incidence of undescended testes in fetuses of rats given monobenzyl phthalate, a major metabolite of butyl benzyl phthalate. Reprod Toxicol 17:407–412.

Fisher JS. 2004. Environmental anti-androgens and male reproductive health: focus on phthalates and testicular dysgenesis syndrome. Reproduction 127:305–315.

Foster PM, Cattley RC, Mylchreest E. 2000. Effects of di-*n*-butyl phthalate (DBP) on male reproductive development in the rat: implications for human risk assessment. Food Chem Toxicol 38:S97–S99.

Foster PM, Lake BG, Thomas LV, Cook MW, Gangolli SD. 1981. Studies on the testicular effects and zinc excretion produced by various isomers of monobutyl-*o*-phthalate in the rat. Chem Biol Interact 34:233–238.

Foster PM, McIntyre BS. 2002. Endocrine active agents: implications of adverse and non-adverse changes. Toxicol Pathol 30:59–65.

Foster PM, Thomas LV, Cook MW, Gangolli SD. 1980. Study of the testicular effects and changes in zinc excretion produced by some *n*-alkyl phthalates in the rat. Toxicol Appl Pharmacol 54:392–398.

Gray LE Jr, Foster PMD. 2003. Significance of experimental studies for assessing adverse effects of endocrine-disrupting chemicals. Pure Appl Chem 75:2125–2141.

Gray LE Jr, Ostby J, Furr J, Price M, Veeramachaneni DNR, Parks L. 2000. Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. Toxicol Sci 58:350–365.

Hauser R, Meeker JD, Park S, Silva MJ, Calafat AM. 2004. Temporal variability of urinary phthalate metabolite levels in men of reproductive age. Environ Health Perspect 112:1734–1740.

Hoppin JA, Brock JW, Davis BJ, Baird DD. 2002. Reproducibility of urinary phthalate metabolites in first morning urine samples. Environ Health Perspect 110:515–518.

Hornung RW, Reed LD. 1990. Estimation of average concentration in the presence of nondetectable values. Appl Occup Environ Hyg 5:46–51.

McIntyre BS, Barlow NJ, Foster PM. 2001. Androgen-mediated development in male rat offspring exposed to flutamide *in utero*: permanence and correlation of early postnatal changes in anogenital distance and nipple retention with malformations in androgen-dependent tissues. Toxicol Sci 62:236–249.

McKee RH, Toxicology Research Task Group. 2004. Phthalate exposure and early thelarche. Environ Health Perspect 112:A541–A543.

Murature DA, Tang SY, Steinhardt G, Dougherty RC. 1987. Phthalate esters and semen quality parameters. Biomed Environ Mass Spectrom 14:473–477.

Mylchreest E, Wallace DG, Cattley RC, Foster PMD. 2000. Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to di(*n*-butyl) phthalate during late gestation. Toxicol Sci 55:143–151.

Nagao T, Ohta R, Marumo H, Shindo T, Yoshimura S, Ono H. 2000. Effect of butyl benzyl phthalate in Sprague-Dawley rats after gavage administration: a two-generation reproductive study. Reprod Toxicol 14:513–532.

Nakahara H, Shono T, Suita S. 2003. Reproductive toxicity evaluation of dietary butyl benzyl phthalate (BBP) in rats. Fukuoka Igaku Zasshi 94:331–337.

Neumann F. 1976. Pharmacological and endocrinological studies on anabolic agents. Environ Qual Saf 5(suppl)253–264.

Parks LG, Ostby JS, Lambright CR, Abbott BD, Klinefelter GR, Barlow NJ, et al. 2000. The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. Toxicol Sci 58:339–349.

Phillip M, De Boer C, Pilpel D, Karplus M, Sofer S. 1996. Clitoral and penile sizes of full term newborns in two different ethnic groups. J Pediatr Endocrinol Metab 9:175–179.

Rhees RW, Kirk BA, Sephton S, Lephart ED. 1997. Effects of prenatal testosterone on sexual behavior, reproductive morphology and LH secretion in the female rat. Dev Neurosci 19:430–437.

Salazar-Martinez E, Romano-Riquer P, Yanez-Marquez E, Longnecker MP, Hernandez-Avila M. 2004. Anogenital distance in human male and female newborns: a descriptive, cross-sectional study. Environ Health 3:8; doi:10.1186/1476-069X-3-8 [Online 13 September 2004].

Silva MJ, Barr DB, Reidy JA, Malek NA, Hodge CC, Caudill SP, et al. 2004a. Urinary levels of seven phthalate metabolites in the U.S. population from the National Health and Nutrition Examination Survey (NHANES) 1999–2000. Environ Health Perspect 112:331–338.

Silva MJ, Malek NA, Hodge CC, Reidy JA, Kato K, Barr DB, et al. 2003. Improved quantitative detection of 11 urinary phthalate metabolites in humans using liquid chromatography-atmospheric pressure chemical ionization tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 789:393–404.

Silva MJ, Slakman AR, Reidy JA, Preau JL Jr, Herbert AR, Samandar E, et al. 2004b. Analysis of human urine for fifteen phthalate metabolites using automated solid-phase extraction. J Chromatogr B Analyt Technol Biomed Life Sci 805:161–167.

Skakkebaek NE, Rajpert-De Meyts E, Main KM. 2001. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. Hum Reprod 16:972–978.

Swan SH, Brazil C, Drobnis EZ, Liu F, Kruse RL, Hatch M, et al. 2003. Geographic differences in semen quality of fertile U.S. males. Environ Health Perspect 111:414–420.

Tyl RW, Myers CB, Marr MC, Fail PA, Seely JC, Brine DR, et al. 2004. Reproductive toxicity evaluation of dietary butyl benzyl phthalate (BBP) in rats. Reprod Toxicol 18:241–264.

Vandenbergh JG, Huggett CL. 1995. The anogenital distance index, a predictor of the intrauterine position effects on reproduction in female house mice. Lab Anim Sci 45:567–573.

White CR, Seymour RS. 2005. Allometric scaling of mammalian metabolism. J Exp Biol 208:1611–1619.

The Association between Asthma and Allergic Symptoms in Children and Phthalates in House Dust: A Nested Case–Control Study

Carl-Gustaf Bornehag,[1,2,3] Jan Sundell,[2] Charles J. Weschler,[2,4] Torben Sigsgaard,[5] Björn Lundgren,[1] Mikael Hasselgren,[3] and Linda Hägerhed-Engman[1]

[1]Swedish National Testing and Research Institute, Borås, Sweden; [2]Technical University of Denmark, Lyngby, Denmark; [3]Public Health Science, Karlstad University, Karlstad, Sweden; [4]University of Medicine and Dentistry New Jersey–Robert Wood Johnson Medical School and Rutgers University, Piscataway, New Jersey, USA; [5]Aarhus University, Aarhus, Denmark

Global phthalate ester production has increased from very low levels at the end of World War II to approximately 3.5 million metric tons/year. The aim of the present study was to investigate potential associations between persistent allergic symptoms in children, which have increased markedly in developed countries over the past three decades, and the concentration of phthalates in dust collected from their homes. This investigation is a case–control study nested within a cohort of 10,852 children. From the cohort, we selected 198 cases with persistent allergic symptoms and 202 controls without allergic symptoms. A clinical and a technical team investigated each child and her or his environment. We found higher median concentrations of butyl benzyl phthalate (BBzP) in dust among cases than among controls (0.15 vs. 0.12 mg/g dust). Analyzing the case group by symptoms showed that BBzP was associated with rhinitis (p = 0.001) and eczema (p = 0.001), whereas di(2-ethylhexyl) phthalate (DEHP) was associated with asthma (p = 0.022). Furthermore, dose–response relationships for these associations are supported by trend analyses. This study shows that phthalates, within the range of what is normally found in indoor environments, are associated with allergic symptoms in children. We believe that the different associations of symptoms for the three major phthalates—BBzP, DEHP, and di-*n*-butyl phthalate—can be explained by a combination of chemical physical properties and toxicologic potential. Given the phthalate exposures of children worldwide, the results from this study of Swedish children have global implications. *Key words:* **allergy, asthma, BBzP, children, DEHP, homes, phthalates.** *Environ Health Perspect* **112:1393–1397 (2004). doi:10.1289/ehp.7187 available via** *http://dx.doi.org/* **[Online 15 July 2004]**

Airborne phthalate esters are present at detectable levels across the surface of Earth. They were first identified in outdoor urban air (Cautreels and Van Cauwenberghe 1976a, 1976b) and subsequently have been recognized as global pollutants (Atlas and Giam 1981; Giam et al. 1978) and major constituents of indoor air (Weschler 1980, 1984). Their presence in outdoor and indoor environments reflects their large emission rates coupled with moderate atmospheric lifetimes. The total global consumption of phthalate esters is estimated to exceed 3.5 million metric tons/year, with di(2-ethylhexyl) phthalate (DEHP) constituting roughly 50% of the market share (Cadogan and Howick 1996). Consumption of di-*n*-butyl phthalate (DnBP) and *n*-butyl benzyl (BBzP) phthalate is smaller but still quite large (> 100,000 metric tons/year each) (Cadogan and Howick 1996). Although DEHP plasticizes numerous products, roughly 95% of the current production is used in polyvinyl chloride (PVC) (National Toxicology Program 2003), where it typically constitutes 30% of PVC by weight (Cadogan and Howick 1996; Kavlock et al. 2002b). DnBP is used in latex adhesives, in nail polish and other cosmetic products, as a plasticizer in cellulose plastics, as a solvent for certain dyes, and, to a lesser extent than DEHP, as a plasticizer in PVC (Kavlock et al. 2002c). BBzP is a plasticizer for vinyl tile, carpet tiles, and artificial leather and is also used in certain adhesives (Kavlock et al. 2002a).

Research groups have assessed the exposures of various populations to phthalate esters by using their metabolites in human urine as biomarkers [Barr et al. 2003; Blount et al. 2000; Centers for Disease Control and Prevention (CDC) 2003; Koch et al. 2003]. The biomarker results translate to daily exposures for DnBP, BBzP, and DEHP of 1.5, 0.88, and 0.71 μg/kg/day in the United States (Kohn et al. 2000); 0.95, 0.71, and 0.84 μg/kg/day in the United States (derived from data from Barr et al. 2003, their Table 1, using the procedure outlined by Kohn et al. 2000); and 5.22, 0.60, and 13.8 μg/kg/day in Germany (Koch et al. 2003). These findings confirm the relatively large daily exposure to phthalates in industrialized countries. Although the dominant route of exposure to DnBP, BBzP, and DEHP is thought to be via ingestion (Fromme et al. 2004; Kavlock et al. 2002a, 2002b, 2002c), few if any population-based data are available to support this statement. Indeed, a recent study has demonstrated associations between phthalate concentrations in inhaled air and urinary monoester metabolites (Adibi et al. 2003).

The incidence of asthma and allergy has increased throughout the developed world over the past 30 years (Beasley et al. 2003). The short interval over which it has occurred implies that the increase is caused by changes in environmental exposures rather than genetic changes (Etzel 2003; Strachan 2000). Changes in indoor environments warrant special attention because indoor air constitutes a dominant exposure route. Increased exposures to allergens and/or adjuvants (enhancing factors) may each be partially responsible for the increase. Multidisciplinary reviews of the scientific literature on associations between indoor exposures and asthma and allergies (Ahlbom et al. 1998; Andersson et al. 1997; Bornehag et al. 2001; Schneider et al. 2003; Wargocki et al. 2002) indicate that the underlying causal factors responsible for these increases remain unknown.

The use of plasticized products and, consequently, exposures to phthalate esters have increased dramatically since the end of World War II. Phthalate esters have been suggested to act as either allergens or adjuvants (Jaakkola et al. 1999; Oie et al. 1997). Several recent studies have examined the ability of different phthalate esters to function as adjuvants in BALB/c mice injected with a known antigen. DEHP displayed an adjuvant effect with immunoglobulin G1 at a concentration of 2,000 mg/mL after both one and two boosters (Larsen et al. 2001b). In contrast, DnBP only showed an adjuvant effect with immunoglobulin G1 after the second booster (Larsen et al. 2002), and BBzP showed no adjuvant effect (Larsen et al. 2003). Consistent with these results, the monoester of DEHP showed an adjuvant effect whereas the monoesters of DnBP and BBzP did not (Larsen et al. 2001a).

The present study is a nested case–control study on 198 symptomatic children and 202 healthy controls, including detailed clinical examinations by physicians in parallel

Address correspondence to C.G. Bornehag, Public Health Sciences, Karlstad University, 651 88 Karlstad, Sweden. Telephone: +46-54-700-25-40. Fax: +46-54-700-25-23. E-mail: carl-gustaf.bornehag@kau.se

The study was supported by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (Formas), Swedish Asthma and Allergy Association's Research Foundation, the Swedish Foundation for Health Care Sciences and Allergy Research, and European Council of Plasticizers and Intermediates.

The authors declare they have no competing financial interests.

Received 15 April 2004; accepted 15 July 2004.

with extensive inspections and measurements within the subjects' homes. The cases and controls were selected from the first phase (Dampness In Buildings and Health, phase I), which was a cross-sectional questionnaire soliciting health and environmental information regarding all 14,077 children 1–6 years of age in the county of Värmland, Sweden; responses were obtained for 10,852 (Bornehag et al. 2003).

The aim of the present study was to investigate potential associations between persistent allergic symptoms in children and the concentrations of different phthalates in dust collected from their homes.

Materials and Methods

Inclusion criteria for cases and controls. The selection criteria for the cases (Dampness In Buildings and Health, phase II) were as follows: *a*) in the initial questionnaire, reports of at least two incidents of eczema, or wheezing or rhinitis without a cold, during the preceding 12 months; and *b*) in the follow-up questionnaire 1.5 years later, at least two of three possible symptoms reported. Inclusion criteria for the controls were *a*) no symptoms in the first questionnaire and *b*) no symptoms in the follow-up questionnaire. For both groups they had to *c*) not have rebuilt their homes because of moisture problems and *d*) not have changed residence since the first questionnaire. All children with at least two symptoms in the first questionnaire were invited to participate in the case–control study (n = 1,056, corresponding to 9.7% of the total population). In the first questionnaire, 5,303 (48.9%) reported no airway, eye, nose, or skin symptoms. Of these, 1,100 children were randomly selected and invited to participate in the case–control study. This process ultimately yielded 198 cases and 202 controls.

Families were more inclined to participate if the child was reported to have more symptoms, if there was no smoking in the family, and if they belonged to a higher socioeconomic group.

Medical examination. The medical examination of the 400 children (3–8 years of age) was performed during the same 2 weeks that the technical investigations of the homes, including dust collection, were carried out. Medical doctors examined the children and took a detailed history of each child. Blood samples were drawn from 387 children and screened for common allergens (Phadiatop, Pharmacia & Upjohn Diagnostics, Uppsala, Sweden), timothy, birch, mugworth, cat, horse, dog, house dust mites (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), and one mold (*Cladosporium*).

Physicians' diagnoses of the children agreed well with the case–control status as reported in the questionnaire. All children with obvious asthma were found among cases, whereas 10 cases were found among controls (two children with rhinitis and eight children with eczema). Furthermore, 13 cases were found to be misclassified. In the analyses regarding case–control status, the study design has been used; that is, the 23 (10 plus 13) misclassified children have not been reclassified.

Building investigations. There were 10 pairs of siblings among the 400 children; hence, they lived in 390 buildings. Between October 2001 and April 2002, six professional inspectors performed visual inspections and indoor air quality assessments, including dust sampling, in these 390 dwellings. During these investigations, a preestablished checklist was followed regarding building characteristics, mold and water damages, surface materials, and other building-related items.

Phthalates in dust. Samples of dust from 390 homes were collected from molding and shelves in the children's bedroom. The dust was collected on 90-mm membrane filters in holders made of styrene-acrylonitrile polymer mounted on a sampler made of polypropylene (VacuuMark disposable nozzle; Petersen Bach, Bjerringbro, Denmark) connected to a vacuum cleaner. The filter was weighed before and after sampling under controlled conditions. Conditioning the filters before weighing (23°C, 50% relative humidity) was critical to obtaining reproducible filter weights. From the 390 homes there were 9 missing samples, 13 samples with errors in the laboratory analysis, and 6 samples with a negative dust weight. Consequently, there were 362 valid samples. Only filters with a reliably measurable net increase in weight (≥ 25 mg) were included in the present analysis; 346 of the 362 dust samples met this criterion.

The dust samples were extracted in precleaned 10-mL glass vials for 30 min using 2 mL dichloromethane. This procedure was repeated, and the two extracts were then combined and transferred to 3-mL autosampler vials. Aliquots from these vials were injected into either a gas chromatograph/mass selective detector (GC/MSD) for phthalate identification or a GC/flame ionization detector for quantitation. GC was performed using a 25-m capillary column (HP 1C; Agilent, Folsom, CA, USA; inner diameter, 0.2 mm; stationary phase, polydimethyl siloxane). The injector temperature was 280°C; column temperature started at 100°C for 3 min and then increased at 8°C/min to 300°C, which was maintained for 20 min. The detector temperature and transfer line to the MSD were maintained at 280°C. The analytical and field sampling techniques were tested in a preliminary study that found only limited influence from background contributions to the analyzed samples. In the present study, field blanks have indicated no significant background contributions. The dust concentrations (milligram per gram dust) of six phthalates were determined: diethyl phthalate (DEP), diisobutyl phthalate (DIBP), DnBP, BBzP, DEHP, and diisononyl phthalate (DINP).

Statistical method. The concentrations of phthalates in the dust were log-normally distributed. Hence, analyses of potential associations between concentrations of phthalates in dust and health outcomes have been conducted using nonparametric tests (Mann-Whitney U-test). Log-transformed, normally distributed concentrations were tested with parametric tests (t-test). The concentrations are reported as medians, as arithmetic means, and as geometric means with 95% confidence intervals (CIs). The CIs were calculated with a back-transform of mean log ± 2 × SE. Dose–response relationships were tested by factoring the phthalate concentrations into quartiles and using both uni- and multivariate logistic regression analyses. Adjustments have been made for environmental tobacco smoke as well as sex and age of the child, because these have been associated with asthma and allergic symptoms. Adjustments for type of building were made, because living in a privately owned single-family house was a selection factor for both cases and controls (Bornehag et al., unpublished data). Indeed, cases and controls lived mainly in single-family houses (88.7%). Furthermore, the frequency of PVC as flooring material was lower in single-family houses than in multifamily houses (51.6 vs. 71.8%). Adjustments for the construction period of the building and self-reported water leakage in the home during the previous 3 years were made because these are associated with the concentrations of phthalates in dust. Finally, adjustments were made for exposure to other phthalates. Multiple logistic regressions were performed by a backward elimination technique where only significant variables were included in the final model. The analyses were considered statistically significant when $p < 0.05$.

The study was approved by the local ethics committee.

Results

Compared with other types of flooring materials, PVC flooring in the child's bedroom was positively associated with case status [adjusted odds ratio (OR), 1.59; 95% CI, 1.05–2.41].

Phthalates in dust. Results are presented in Tables 1–3 and Figure 1. In Tables 1 and 2, median phthalate dust concentrations are reported for data sets that include all valid samples with a reliably measurable net increase in weight (346 of 390 homes), and geometric mean concentrations are reported for data sets that exclude samples with phthalate dust concentrations less than the detection limit. (If, instead, nondetects were

assigned concentrations of one-half the detection limit, then for phthalates with a large number of nondetects, their dust concentrations would no longer be log-normally distributed.) The geometric mean concentrations of BBzP and DEHP were higher in bedrooms with PVC flooring than in bedrooms without such flooring [BBzP: 0.208 (n = 164) vs. 0.147 (n = 107) mg/g dust; DEHP: 0.994 (n = 186) vs. 0.638 (n = 155) mg/g dust; both $p < 0.001$ by t-test]. DEP, DIBP, DnBP, and DINP were not associated with PVC flooring.

Association between phthalates in dust and health effects. Cases had a higher concentrations of BBzP in the dust samples from the children's bedrooms than did the controls in parametric as well as in nonparametric tests (Table 1). Cases with physician-diagnosed rhinitis or eczema had higher BBzP concentrations in the bedroom dust compared with controls (Table 2). Furthermore, cases with doctor-diagnosed asthma had higher DEHP concentrations in the dust compared with controls. In analyses restricted to single-family and row houses, the same associations were found (data not shown).

In an analysis restricted to homes with PVC flooring in the child's bedroom (n = 189), the median BBzP concentration was significantly higher among cases compared with controls (0.21 vs. 0.16 mg/g dust, respectively; Mann-Whitney U-test, p = 0.042), and BBzP was associated with rhinitis and eczema (Table 2). Such differences between cases and controls were not observed for DEHP.

BBzP concentrations in the highest quartile were associated with an increased risk of being a "case child" (Table 3). The same association was found after adjusting for possible confounders. Table 3 also shows associations between phthalates in dust and doctor-diagnosed asthma, rhinitis, or eczema. A dose–response relationship was found between concentrations of BBzP in dust and doctor-diagnosed rhinitis and eczema in both crude and adjusted analyses. For DEHP, a dose–response relationship was found for asthma in both crude and adjusted analyses, as well as in analysis restricted to single-family houses (data not shown for the latter).

Specific immunoglobulin E in blood. Figure 1 presents the concentration of phthalates in dust among cases and controls with and without specific immunoglobulin E in blood (i.e., atopics and nonatopics). Within the group of cases, the highest geometric mean concentrations of BBzP were found in dust from the bedrooms of atopics. However, when comparing cases with and without atopy, the difference was not statistically significant (p = 0.564).

Discussion

In the present study we found associations between dust concentrations of specific phthalate esters and asthma, rhinitis, and eczema. As shown in Tables 2 and 3, BBzP is significantly associated with doctor-diagnosed rhinitis and eczema, whereas DEHP is significantly associated with doctor-diagnosed asthma. Interestingly, no such associations are found for DnBP despite the fact that the median concentrations of BBzP and DnBP in the settled dust were comparable (0.150 vs. 0.135 mg/g; Table 1). Hence, these three phthalates display strikingly different associations between their dust concentrations and the health outcomes monitored in this study. From a physical chemistry viewpoint, DnBP, BBzP, and DEHP are significantly different from one another; they possess different vapor pressures, polarities, water solubilities, and octanol/air partition coefficients. For example, the vapor pressures of DnBP and BBzP are two orders of magnitude greater than that of DEHP. This means that greater fractions of DnBP and BBzP are in the gas phase as

Table 1. Concentrations of phthalates in surface dust from children's bedrooms.

		Median (arithmetic mean) concentration of phthalates (mg/g dust)									
							All	Cases		Controls	
Phthalate	No. of homes[a]	All samples (n = 346)	Cases (n = 175)[b]	Controls (n = 177)[b]	U-test[c] (p-value)	No. of homes[d]	samples GM conc	No.	GM conc [(95% CI) mg/g dust]	No.	GM conc [(95% CI) mg/g dust]	t-Test[e] (p-value)
DEP	346	0.000 (0.031)	0.000 (0.046)	0.000 (0.018)	0.628	47	0.073	22	0.102 (0.049–0.211)	26	0.058 (0.035–0.097)	0.200
DIBP	346	0.045 (0.097)	0.042 (0.102)	0.048 (0.092)	0.424	290	0.056	141	0.058 (0.048–0.070)	154	0.055 (0.046–0.065)	0.635
DnBP	346	0.150 (0.226)	0.150 (0.228)	0.149 (0.220)	0.914	308	0.174	158	0.171 (0.152–0.193)	154	0.178 (0.157–0.201)	0.639
BBzP	346	0.135 (0.319)	0.152 (0.472)	0.118 (0.163)	0.014	272	0.181	139	0.209 (0.180–0.244)	137	0.157 (0.139–0.178)	0.004
DEHP	346	0.770 (1.310)	0.828 (1.384)	0.723 (1.229)	0.160	343	0.789	173	0.836 (0.724–0.964)	176	0.741 (0.643–0.855)	0.232
DINP	346	0.041 (0.639)	0.000 (0.671)	0.047 (0.589)	0.848	175	0.451	87	0.453 (0.352–0.583)	90	0.446 (0.351–0.566)	0.925

Abbreviations: conc, concentration; GM, geometric mean.
[a]Number of homes with a dust sample weight > 25 mg. [b]The sum of cases and controls is 352 because, among the 346 bedrooms, there were six bedrooms shared by siblings. [c]Mann-Whitney U-test. [d]Number of homes with a dust sample weight > 25 mg and a phthalate concentration greater than the detection limit (0.040 mg/g dust for DnBP, BBzP, and DEHP). [e]Test of the difference between cases and controls made on mean log-transformed concentration.

Table 2. Concentration of phthalates (BBzP and DEHP) in surface dust for case children with a doctor-diagnosed disease compared with controls.

		Cases[a]		Controls			Cases		Controls		
Phthalate	Disease	No.	Median conc (mg/g dust)	No.	Median conc (mg/g dust)	U-test[b] (p-value)	No.	GM conc [(95% CI) mg/g dust]	No.	GM conc [(95% CI) mg/g dust]	t-Test[c] (p-value)
All homes											
BBzP	Asthma	106	0.152	177	0.118	0.064	82	0.219 (0.177–0.270)	137	0.157 (0.139–0.178)	0.005
	Rhinitis	79	0.181	177	0.118	0.007	65	0.237 (0.185–0.304)	137	0.157 (0.139–0.178)	0.001
	Eczema	115	0.181	177	0.118	0.001	95	0.224 (0.186–0.269)	137	0.157 (0.139–0.178)	0.001
DEHP	Asthma	106	0.899	177	0.723	0.008	106	0.966 (0.807–1.156)	176	0.741 (0.643–0.855)	0.022
	Rhinitis	79	0.783	177	0.723	0.383	78	0.811 (0.638–1.030)	176	0.741 (0.643–0.855)	0.510
	Eczema	115	0.844	177	0.723	0.111	115	0.855 (0.721–1.014)	176	0.741 (0.643–0.855)	0.207
Homes with PVC flooring in the child's bedroom											
BBzP	Asthma	59	0.195	82	0.159	0.168	52	0.237 (0.177–0.316)	71	0.177 (0.148–0.212)	0.076
	Rhinitis	45	0.216	82	0.159	0.008	43	0.265 (0.192–0.366)	71	0.177 (0.148–0.212)	0.018
	Eczema	70	0.216	82	0.159	0.003	66	0.257 (0.204–0.324)	71	0.177 (0.148–0.212)	0.011
DEHP	Asthma	59	1.006	82	0.855	0.149	59	1.148 (0.904–1.459)	82	0.938 (0.752–1.169)	0.228
	Rhinitis	45	0.792	82	0.855	0.924	44	1.040 (0.771–1.403)	82	0.938 (0.752–1.169)	0.586
	Eczema	70	0.904	82	0.855	0.379	70	1.045 (0.845–1.291)	82	0.938 (0.752–1.169)	0.491

Abbreviations: conc, concentration; GM, geometric mean.
[a]Cases with doctor diagnosed disease (asthma, rhinitis, or eczema). [b]Mann-Whitney U-test. [c]Test of the difference between cases and controls made on mean log-transformed concentration.

opposed to the condensed phase (i.e., associated with dust and airborne particles). We estimate that, for a particle concentration of 20 μg/m^3, > 80% of airborne DnBP and > 80% of airborne BBzP are in the gas phase, whereas > 85% of airborne DEHP is associated with airborne particles (Weschler 2003). The deposition of a compound in the respiratory tract is strongly influenced by whether it is present in the gas phase or associated with airborne particles. Furthermore, as a consequence of their inherent chemical differences, DnBP, BBzP, and DEHP, as well as their monoester metabolites, produce different effects in a mouse model (Larsen et al. 2001a, 2001b, 2002, 2003). Furthermore, each of these phthalates has its distinct human metabolic pathway (Barr et al. 2003). We suspect that the different relative distributions between the gas and condensed phases, coupled with different toxicologic and pharmacokinetic behaviors, contribute to the fact that DnBP, BBzP, and DEHP are associated with different health outcomes (i.e., DnBP, no associations; BBzP, skin and mucosa symptoms; DEHP, lower airway symptoms).

In the present study there is a general association between PVC flooring and case status (OR, 1.59). Both BBzP and DEHP correlate with the amount of PVC flooring in the subjects' homes. However, these two phthalates are not associated with health effects simply because they are associated with PVC flooring. This conclusion is supported by a number of observations: First, specific associations between BBzP and DEHP dust concentrations and doctor-diagnosed diseases (Table 3) are more pronounced than associations between PVC flooring and such diseases. Second, although BBzP and DEHP dust concentrations do correlate, the correlation is weak (R = 0.52), and they are associated with different health effects. Third, in a restricted analysis, including only homes with PVC flooring, higher concentrations of BBzP were found in dust from case homes than in that from control homes.

The reported concentrations of phthalates in the bedroom dust (Table 1) are consistent with those reported in other studies. In dust samples from 120 U.S. homes located on Cape Cod, Massachusetts (Rudel et al. 2003), the median concentrations were 0.34, 0.045, and 0.020 mg/g dust for DEHP, BBzP, and DnBP, respectively. In a study of 59 apartments in Berlin, Germany (Fromme et al. 2004), the median concentrations were 0.70, 0.030, and 0.047 mg/g dust for DEHP, BBzP, and DnBP. Clausen et al. (2003) measured mean DEHP concentrations of 3.2 mg/g dust in 15 Danish schools and 0.86 mg/g dust for 23 Danish homes. Oie et al. (1997) reported mean concentrations of 0.64 mg DEHP/g dust and 0.11 mg BBzP/g dust for 38 homes in Norway. Pohner et al. (1997) reported a 95th percentile DEHP concentration of 2.0 mg/g dust for 272 German homes, whereas another German study on 286 homes reported a 95th percentile DEHP concentration of 2.6 mg/g dust (Butte et al. 2001).

Regarding atopic status and its association with phthalate dust concentrations, the chosen study design is not optimal. Because there were only 16 atopic controls, the power of the analysis on atopic children is limited. On the other hand, our findings could be interpreted to mean that the mechanism is of a nonimmunologic nature (e.g., exposure increases the risk for irritation).

To identify potential selection biases in the study group, we obtained information for all invited families from the first cross-sectional questionnaire. This revealed that the final study group contained significantly more single-family houses than the eligible population. Adjusting and restricting the analyses have addressed this problem. There was no selection bias regarding PVC flooring because included and nonincluded cases and controls reported about the same frequency of occurrence of PVC flooring in the child's bedroom (Bornehag et al., unpublished data). Furthermore, 10 controls and 13 cases were misclassified when comparing self-reported symptoms and doctors diagnoses. However, when these children were excluded from the analyses, the reported associations remained. Finally, to be included as a "case," a child was required to have at least two symptoms. Consequently, this study was not fine-tuned to examine associations between building factors and single symptoms (i.e., asthma, rhinitis, or eczema). However, even if the design is suboptimal, meaning it was more difficult to find associations between single symptoms and exposures, the association between selected building factors and single symptoms is meaningful and possibly underestimates true associations.

Table 3. Crude and adjusted ORs (95% CIs) between phthalates (BBzP and DEHP) in surface dust and case status or doctor-diagnosed disease.

	Quartile				
Group[a]	1 (ref; n = 88)	2 (n = 88)	3 (n = 88)	4 (n = 88)	p-Value[b]
BBzP					
Ranges (mg BBzP/g dust)	0.00–0.05	0.05–0.13	0.13–0.25	0.25–45.55	
Crude analysis					
Case status	1.0	0.69 (0.38–1.26)	1.00 (0.55–1.81)	2.01 (1.10–3.69)	0.012
Asthma	1.0	0.63 (0.31–1.27)	0.59 (0.45–1.76)	1.92 (0.98–3.79)	0.039
Rhinitis	1.0	0.85 (0.38–1.89)	1.12 (0.51–2.47)	2.69 (1.26–5.76)	0.006
Eczema	1.0	0.74 (0.36–1.52)	1.44 (0.73–2.81)	2.52 (1.26–5.00)	0.002
Adjusted[c] analysis					
Case status	1.0	0.77 (0.40–1.46)	1.01 (0.53–1.90)	1.95 (1.02–3.74)	—
Asthma	1.0	0.67 (0.33–1.38)	0.88 (0.43–1.80)	1.87 (0.92–3.81)	—
Rhinitis	1.0	1.03 (0.44–2.39)	1.23 (0.53–2.88)	3.04 (1.34–6.89)	—
Eczema	1.0	0.84 (0.40–1.76)	1.45 (0.71–2.97)	2.56 (1.24–5.32)	—
DEHP					
Ranges (mg DEHP/g dust)	0.00–0.46	0.46–0.77	0.77–1.30	1.30–40.46	
Crude analysis					
Case status	1.0	0.91 (0.50–1.65)	1.05 (0.58–1.89)	1.44 (0.80–2.61)	0.199
Asthma	1.0	1.11 (0.53–2.31)	1.51 (0.74–3.07)	2.36 (1.17–4.75)	0.009
Rhinitis	1.0	1.12 (0.53–2.36)	0.96 (0.44–2.11)	1.55 (0.73–3.28)	0.331
Eczema	1.0	1.00 (0.50–1.97)	1.35 (0.70–2.62)	1.50 (0.76–2.96)	0.161
Adjusted[c] analysis					
Case status	1.0	NS	NS	NS	—
Asthma	1.0	1.56 (0.70–3.46)	2.05 (0.94–4.47)	2.93 (1.36–6.34)	—
Rhinitis	1.0	NS	NS	NS	—
Eczema	1.0	NS	NS	NS	—

—, no analyses have been done because linear-by-linear association cannot be done in a multivariate manner; NS, not significant in model, using backward elimination; ref, reference.
[a]Case status and subgroups with asthma, rhinitis, or eczema compared with controls. [b]Linear-by-linear association. [c]Adjustments made for sex, age, smoking at home, type of building, construction period, self-reported flooding during preceding 3 years, and the other phthalate variable (in quartiles), using backward elimination method; only significant variables were included in the final model.

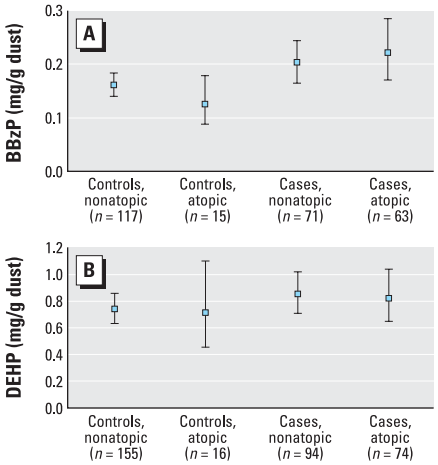


Figure 1. Geometric mean concentrations (95% CIs) of phthalates (*A*), BBzP, and (*B*), DEHP in surface dust from bedrooms of nonatopic and atopic children.

The reported analyses are based on samples with a weight > 25 mg. However, when including all samples (n = 362), the reported associations between exposure and symptoms remained or became stronger (data not shown).

Koo et al. (2002) present weak associations between exposure estimates for different phthalate esters, based on their urinary biomarkers, and the level of education, family income, and residency (urban or rural) in a reference U.S. population. Given that study, one might speculate that the associations reported in the present study are driven by demographic factors. However, in contrast to the United States, where 22.4% of the children live in households with incomes < 50% of the national median, in Sweden only 2.6% of the children live in such households (Unicef 2000). Additionally, the association in our study holds when the analysis is restricted to single-family houses; such homes have an even more homogeneous socioeconomic status. Hence, different demographic factors between cases and controls appear to be an unlikely explanation for the associations observed in the present study. Furthermore, given that the dust concentrations of DnBP, BBzP, and DEHP display quite different associations with different symptoms, the associations reflect a biologic response rather than just lifestyle or demographic factors associated with an increased use of plasticized materials.

This study demonstrates associations between BBzP and DEHP concentrations in dust and selected allergies and asthma. Although multiple factors likely are responsible for the increases in allergies and asthma that have been documented in developed countries over the past 30 years, it is striking that these increases have occurred during a period when plasticized products have become ubiquitous in the homes, schools, and workplaces of the developed world.

References

Adibi JJ, Perera FP, Jedrychowski W, Camann DE, Barr D, Jacek R, et al. 2003. Prenatal exposures to phthalates among women in New York City and Krakow, Poland. Environ Health Perspect 111:1719–1722.

Ahlbom A, Backman A, Bakke J, Foucard T, Halken S, Kjellman NIM, et al. 1998. NORDPET. Pets indoors—a risk factor for or protection against sensitization/allergy. A Nordic interdisciplinary review of the scientific literature concerning the relationship between the exposure to pets at home, sensitization and the development of allergy. Indoor Air 8:219–235.

Andersson K, Bakke JV, Bjorseth O, Bornehag CG, Clausen G, Hongslo JK, et al. 1997. TVOC and health in non-industrial indoor environments. Report from a Nordic scientific consensus meeting at Langholmen in Stockholm. Indoor Air 7:78–91.

Atlas EL, Giam CS. 1981. Global transport of organic pollutants—ambient concentrations in the remote marine atmosphere. Science 211:163–165.

Barr DB, Silva MJ, Kato K, Reidy JA, Malek NA, Hurtz D, et al. 2003. Assessing human exposure to phthalates using monoesters and their oxidized metabolites as biomarkers. Environ Health Perspect 111:1148–1151.

Beasley R, Ellwood P, Asher I. 2003. International patterns of the prevalence of pediatric asthma: the ISAAC program. Pediatr Clin North Am 50:539–553.

Blount BC, Silva MJ, Caudill SP, Needham LL, Pirkle JL, Sampson EJ, et al. 2000. Levels of seven urinary phthalate metabolites in a human reference population. Environ Health Perspect 108:979–982.

Bornehag CG, Blomquist G, Gyntelberg F, Jarvholm B, Malmberg P, Nordvall L, et al. 2001. Dampness in buildings and health. Nordic interdisciplinary review of the scientific evidence on associations between exposure to "dampness" in buildings and health effects (NORDDAMP). Indoor Air 11:72–86.

Bornehag CG, Sundell J, Hagerhed L, Janson S. 2003. Pet-keeping in early childhood and airway, nose and skin symptoms later in life. Allergy 58:939–944.

Butte W, Hoffmann W, Hostrup O, Schmidt A, Walker G. 2001. Endocrine disrupting chemicals in house dust: results of a representative monitoring. Gefahrstoffe Reinhaltung Der Luft 61:19–23.

Cadogan DF, Howick CJ. 1996. Plasticizers. In: Kirk-Othmer Encyclopedia of Chemical Technology, Vol 19. New York:John Wiley and Sons, 258–290.

Cautreels W, van Cauwenberghe K. 1976a. Determination of organic-compounds in airborne particulate matter by gas chromatography mass spectrometry. Atmos Environ 10:447–457.

Cautreels W, van Cauwenberghe K. 1976b. Extraction of organic-compounds from airborne particulate matter. Water Air Soil Pollut 6:103–110.

CDC. 2003. Second National Report on Human Exposure to Environmental Chemicals. Atlanta, GA:Centers for Disease Control and Prevention, National Center for Environmental Health.

Clausen PA, Lindeberg Bille RL, Nilsson T, Hansen V, Svensmark B, Bowadt S. 2003. Simultaneous extraction of di(2-ethylhexyl) phthalate and nonionic surfactants from house dust. Concentrations in floor dust from 15 Danish schools. J Chromatogr A 986:179–190.

Etzel RA. 2003. How environmental exposures influence the development and exacerbation of asthma. Pediatrics 112:233–239.

Fromme H, Lahrz T, Piloty M, Gebhart H, Oddoy A, Rüden H. 2004. Occurrence of phthalates and musk fragrances in indoor air and dust from apartments and kindergartens in Berlin (Germany). Indoor Air 14:188–195.

Giam CS, Chan HS, Neff GS, Atlas EL. 1978. Phthalate ester plasticizers: a new class of marine pollutant. Science 199:419–421.

Jaakkola JJ, Oie L, Nafstad P, Botten G, Samuelsen SO, Magnus P. 1999. Interior surface materials in the home and the development of bronchial obstruction in young children in Oslo, Norway. Am J Public Health 89:188–192.

Kavlock R, Boekelheide K, Chapin R, Cunningham M, Faustman E, Foster P, et al. 2002a. NTP Center for the Evaluation of Risks to Human Reproduction: phthalates expert panel report on the reproductive and developmental toxicity of butyl benzyl phthalate. Reprod Toxicol 16:453–487.

Kavlock R, Boekelheide K, Chapin R, Cunningham M, Faustman E, Foster P, et al. 2002b. NTP Center for the Evaluation of Risks to Human Reproduction: phthalates expert panel report on the reproductive and developmental toxicity of di(2-ethylhexyl) phthalate. Reprod Toxicol 16:529–653.

Kavlock R, Boekelheide K, Chapin R, Cunningham M, Faustman E, Foster P, et al. 2002c. NTP Center for the Evaluation of Risks to Human Reproduction: phthalates expert panel report on the reproductive and developmental toxicity of di-*n*-butyl phthalate. Reprod Toxicol 16:489–527.

Koch HM, Drexler H, Angerer J. 2003. An estimation of the daily intake of di(2-ethylhexyl)phthalate (DEHP) and other phthalates in the general population. Int J Hyg Environ Health 206:77–83.

Kohn MC, Parham F, Masten SA, Portier CJ, Shelby MD, Brock JW, et al. 2000. Human exposure estimates for phthalates [Letter]. Environ Health Perspect 108:A440–A442.

Koo JW, Parham F, Kohn MC, Masten SA, Brock JW, Needham LL, et al. 2002. The association between biomarker-based exposure estimates for phthalates and demographic factors in a human reference population. Environ Health Perspect 110:405–410.

Larsen ST, Hansen JS, Thygesen P, Begtrup M, Poulsen OM, Nielsen GD. 2001a. Adjuvant and immuno-suppressive effect of six monophthalates in a subcutaneous injection model with BALB/c mice. Toxicology 169:37–51.

Larsen ST, Lund RM, Damgard Nielsen G, Thygesen P, Poulsen OM. 2001b. Di-(2-ethylhexyl) phthalate possesses an adjuvant effect in a subcutaneous injection model with BALB/c mice. Toxicol Lett 125:11–18.

Larsen ST, Lund RM, Nielsen GD, Thygesen P, Poulsen OM. 2002. Adjuvant effect of di-*n*-butyl-, di-*n*-octyl-, di-iso-nonyl- and di-iso-decyl phthalate in a subcutaneous injection model using BALB/c mice. Pharmacol Toxicol 91:264–272.

Larsen ST, Lund RM, Thygesen P, Poulsen OM, Nielsen GD. 2003. Investigation of the adjuvant and immuno-suppressive effects of benzyl butyl phthalate, phthalic acid and benzyl alcohol in a murine injection model. Food Chem Toxicol 41:439–446.

NTP. 2003. 10th Report on Carcinogens. Research Triangle Park, NC:National Toxicology Program.

Oie L, Hersoug LG, Madsen JO. 1997. Residential exposure to plasticizers and its possible role in the pathogenesis of asthma. Environ Health Perspect 105:972–978.

Pohner A, Simrock S, Thumulla J, Weber S, Wirkner T. 1997. Hintergrundbelastung des hausstaubes von privathauhalten mit mittel- und schwerfluchtigen organischen schadstoffen [in German]. Umwelt Gesundheit 2:79.

Rudel RA, Camann DE, Spengler JD, Korn LR, Brody JG. 2003. Phthalates, alkylphenols, pesticides, polybrominated diphenyl ethers, and other endocrine-disrupting compounds in indoor air and dust. Environ Sci Technol 37:4543–4553.

Schneider T, Sundell J, Bischof W, Bohgard M, Cherrie JW, Clausen PA, et al. 2003. "EUROPART." Airborne particles in the indoor environment. A European interdisciplinary review of scientific evidence on associations between exposure to particles in buildings and health effects. Indoor Air 13:38–48.

Strachan DP. 2000. The role of environmental factors in asthma. Br Med Bull 56:865–882.

Unicef. 2000. A League Table of Child Poverty in Rich Nations. Florence, Italy:United Nations Children's Fund Innocenti Research Centre.

Wargocki P, Sundell J, Bischof W, Brundrett G, Fanger PO, Gyntelberg F, et al. 2002. Ventilation and health in non-industrial indoor environments: report from a European multidisciplinary scientific consensus meeting (EUROVEN). Indoor Air 12:113–128.

Weschler CJ. 1980. Characterization of selected organics in size-fractionated indoor aerosols. Environ Sci Technol 14:428–431.

Weschler CJ. 1984. Indoor-outdoor relationships for nonpolar organic constituents or aerosol particles. Environ Sci Technol 18:648–652.

Weschler CJ. 2003. Indoor/outdoor connections exemplified by processes that depend on an organic compound's saturation vapor pressure. Atmos Environ 37:5451–5453.

Human Breast Milk Contamination with Phthalates and Alterations of Endogenous Reproductive Hormones in Infants Three Months of Age

Katharina M. Main,[1] Gerda K. Mortensen,[1] Marko M. Kaleva,[2] Kirsten A. Boisen,[1] Ida N. Damgaard,[1] Marla Chellakooty,[1] Ida M. Schmidt,[1] Anne-Maarit Suomi,[2] Helena E. Virtanen,[2] Jørgen H. Petersen,[1,3] Anna-Maria Andersson,[1] Jorma Toppari,[1,2] and Niels E. Skakkebæk[1]

[1]University Department of Growth and Reproduction, Rigshospitalet, Copenhagen, Denmark; [2]Departments of Physiology and Pediatrics, University of Turku, Turku, Finland; [3]Department of Biostatistics, University of Copenhagen, Copenhagen, Denmark

Phthalates adversely affect the male reproductive system in animals. We investigated whether phthalate monoester contamination of human breast milk had any influence on the postnatal surge of reproductive hormones in newborn boys as a sign of testicular dysgenesis.

Design: We obtained biologic samples from a prospective Danish–Finnish cohort study on cryptorchidism from 1997 to 2001. We analyzed individual breast milk samples collected as additive aliquots 1–3 months postnatally (n = 130; 62 cryptorchid/68 healthy boys) for phthalate monoesters [mono-methyl phthalate (mMP), mono-ethyl phthalate (mEP), mono-*n*-butyl phthalate (mBP), mono-benzyl phthalate (mBzP), mono-2-ethylhexyl phthalate (mEHP), mono-isononyl phthalate (miNP)]. We analyzed serum samples (obtained in 74% of all boys) for gonadotropins, sex-hormone binding globulin (SHBG), testosterone, and inhibin B.

Results: All phthalate monoesters were found in breast milk with large variations [medians (minimum–maximum)]: mMP 0.10 (< 0.01–5.53 μg/L), mEP 0.95 (0.07–41.4 μg/L), mBP 9.6 (0.6–10,900 μg/L), mBzP 1.2 (0.2–26 μg/L), mEHP 11 (1.5–1,410 μg/L), miNP 95 (27–469 μg/L). Finnish breast milk had higher concentrations of mBP, mBzP, mEHP, and Danish breast milk had higher values for miNP (p = 0.0001–0.056). No association was found between phthalate monoester levels and cryptorchidism. However, mEP and mBP showed positive correlations with SHBG (r = 0.323, p = 0.002 and r = 0.272, p = 0.01, respectively); mMP, mEP, and mBP with LH:free testosterone ratio (r = 0.21–0.323, p = 0.002–0.044) and miNP with luteinizing hormone (r = 0.243, p = 0.019). mBP was negatively correlated with free testosterone (r = –0.22, p = 0.033). Other phthalate monoesters showed similar but nonsignificant tendencies.

Conclusions: Our data on reproductive hormone profiles and phthalate exposures in newborn boys are in accordance with rodent data and suggest that human Leydig cell development and function may also be vulnerable to perinatal exposure to some phthalates. Our findings are also in line with other recent human data showing incomplete virilization in infant boys exposed to phthalates prenatally.

Key words: breast milk, exposure, human, infant, phthalate monoester, reproduction. *Environ Health Perspect* **114:270–276 (2006). doi:10.1289/ehp.8075 available via *http://dx.doi.org/* [Online 7 September 2005]**

Phthalates are chemicals with known endocrine-disrupting effects in rodents. Animal studies suggest that prenatal exposure to certain phthalates, specifically di-butyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP), induces adverse effects on the male fetus that are distinct from effects seen in adult animals. DBP, DEHP and its metabolite mono-2-ethylhexyl phthalate (mEHP), and di-isononyl phthalate (DiNP) show antiandrogenic effects. They alter Leydig cell differentiation and function and thus diminish fetal testosterone production (Borch et al. 2004, 2005; Fisher et al. 2003; Foster et al. 2001; Gray et al. 2000). Animals exposed *in utero* to DEHP show reduced anogenital distance and nipple retention. Additionally, a few animals have atrophic testes, severely reduced sperm production, cryptorchidism, or hypospadias (Jarfelt et al. 2005). These antiandrogenic actions of phthalates have been documented in several animal species (Kavlock et al. 2002a, 2002b).

Because phthalates are present ubiquitously in the environment (e.g., polyvinyl chloride flooring, children's toys, detergents, personal care products) and in diet through food production processes and packaging, humans are continuously exposed. However, few population studies on phthalate levels in humans have been reported, and the significance of exposure for human health is still unknown. Metabolites such as phthalate monoesters are particularly high in urine samples of young women and children with yet-unexplained differences between social classes and ethnic groups (Silva et al. 2004b). Recently, phthalates were also detected in pooled breast milk samples from American women (Calafat et al. 2004) and in infant formula (Latini et al. 2004; Mortensen et al. 2005; Petersen and Breindahl 2000; Shea 2003).

Adverse effects of fetal phthalate exposure of humans may be detectable only in adulthood, and the development of early biomarkers for adverse effects is thus imperative. Newborn boys naturally exhibit a short activation of the pituitary–gonadal axis at approximately 3 months of age (Andersson et al. 1998). This feature can be applied diagnostically in cases of gonadotropin deficiency or testicular malfunction, because patients show a blunted or even absent postnatal hormonal peak (Main et al. 2000).

In this study we aimed to evaluate adverse reproductive effects of exposure to phthalates in newborn boys by correlating reproductive hormone levels at 3 months of age to the concentration of six phthalate monoesters in breast milk, the major source of nutrition for infants worldwide.

Materials and Methods

We obtained breast milk samples from a joint prospective, longitudinal cohort study performed 1997–2001 at Turku University Hospital, Turku, Finland, and the National University Hospital, Rigshospitalet, Copenhagen, Denmark. In this binational study we aimed to establish contemporary prevalence rates and geographic differences for cryptorchidism and hypospadias and evaluate risk factors for genital malformations (lifestyle and exposure) by means of questionnaires and biologic samples (blood samples of mother and child, placentas and one breast milk sample from each mother). The study was prospectively planned by both research groups as a joint venture in 1996. Recruitment, inclusion criteria, and clinical examination of the children—the identification of cases with genital malformations and controls—have been described previously (Boisen et al. 2004). All boys in these two cohort studies were examined clinically at birth and again at 3 months of age

Address correspondence to K.M. Main, University Department of Growth and Reproduction, Section 5064, Rigshospitalet, Blegdamsvej 9, DK-2100, Copenhagen, Denmark. Telephone: 45 3545 5085. Fax: 45 3545 6054. E-mail: katharina.main@rh.hosp.dk

This study was supported by the European Commission (QLK4-CT-1999-01422 and QLK4-CT-2001-00269), The Danish Medical Research Council (9700833, 9700909), the Svend Andersen's and Velux Foundations, The Turku University Central Hospital, and the Academy of Finland. The sponsors had no part in study design, data collection, analysis, interpretation, or writing of the manuscript. The article does not represent the opinion of the European Commission, which is not responsible for any use that might be made of data appearing therein.

The authors declare they have no competing financial interests.

Received 2 March 2005; accepted 7 September 2005.

for signs of cryptorchidism. Standardization of the clinical examination procedures was achieved by repetitive workshops. Exposure measurements in biologic samples were prospectively planned to include persistent and nonpersistent chemicals (European Commission grant QLK4-CT-2001-00269).

From the total biobank of breast milk samples, we included 65 samples from each country for phthalate measurements (total n = 130), the number being determined by the funding obtained for chemical analyses. These samples represent 29/33 Danish/Finnish boys with cryptorchidism (unilateral or bilateral) either only at birth (25/8), or at birth and at 3 months of age (4/25). Thirty-six Danish and 32 Finnish control boys without cryptorchidism at any examination were included. In Denmark, these control boys were selected from the entire birth cohort at random (case-cohort design). In Finland, control boys were selected prospectively by a case–control design in which boys with cryptorchidism were matched at birth with controls for maternal parity, smoking (yes/no), diabetes (yes/no), gestational age (± 7 days), and date of birth (± 14 days). This design was chosen in Finland because of lack of sufficient funding to follow the entire cohort through infancy. We calculated weight for gestational age as percent deviation from the expected mean (Marsál et al. 1996), –22% being equivalent to –2 SDs. Three boys with cryptorchidism and 1 control were born small for gestational age (< –22%); 5 boys with cryptorchidism and 3 controls were born prematurely (< 37 weeks of gestation).

The study was conducted according to the Helsinki II declaration (World Medical Association 2004), after informed oral and written consent of the parents. It was approved by the ethical committees in both countries (Joint Commission on Ethics of the Turku University and the Turku University Central Hospital, Turku, Finland; and the Ethical Committees of Copenhagen and Frederiksberg County, Cophenhagen, Denmark; and the Danish Data Protection Agency, Cophenhagen, Denmark).

Each mother collected one breast milk sample. Because we wished to assess the average exposure to phthalates during the time period preceding the endogenous hormone surge, this sample consisted of many small aliquots collected over successive infant feedings over several weeks up to a maximum sample volume of 200 mL. For storage of the breast milk sample, 250-mL Pyrex glass bottles (Bibby Sterilin, Staffordshire, U.K.) with Teflon-coated caps were given to the mothers at birth. Mothers were instructed orally and in writing to feed the baby first and then to sample milk aliquots (hind milk), starting from 1 month after birth. This start point was chosen after discussion with the ethical committee for human subject studies to ensure that breastfeeding had been well established beforehand. Mothers were instructed to collect samples into a glass container or porcelain cup, avoiding the use of mechanical breast pumps, if possible. Breast milk was frozen consecutively in household freezers in a single glass bottle as additive aliquots and delivered frozen to the hospital at the 3-month examination. At the hospital, samples were stored at –20°C until analysis. Only breast milk samples with total volumes > 50 mL were included in the analyses to ensure that all prospectively planned chemical analyses could be performed. For 57 Danish mothers, information on breast pump use was obtained at sample delivery; 26 (46%) had used a pump on one or more occasions during sample collection for the study.

Venous nonfasting blood samples (4 mL) were collected from the same boys whose breast milk samples were used for analysis of phthalate monoesters. Blood samples were drawn on the day the breast milk sample was delivered to the hospital. The boys were median 3.01 (range, 2.43–4.08) months of age. The success rate of venipuncture was 74% (total n = 96; cryptorchid, n = 50; normal boys, n = 46). After clotting, the blood samples were centrifuged, and the sera were separated and stored at –20°C until analyzed.

All blood samples were analyzed as duplicates and blinded for the technician at one laboratory (Rigshospitalet, Denmark). Each run contained blood samples of both cryptorchid and healthy boys from both Finland and Denmark to minimize any effect of interassay variation.

Serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), and sex hormone–binding globulin (SHBG) were analyzed by time-resolved immunofluorometric assays (Delfia, Wallac Inc., Turku, Finland). Detection limits were 0.06 and 0.05 IU/L for FSH and LH, respectively, and 0.23 nmol/L for SHBG. The intra- and interassay coefficients of variation (CV) were < 5% in both gonadotropin assays and < 6% in the SHBG assay. Serum testosterone was measured by radioimmunoassay (Coat-a-Count; Diagnostic Products Corp., Los Angeles, CA, USA), with a detection limit of 0.23 nmol/L; intra- and interassay CVs were < 10%. Free testosterone index was calculated from testosterone and SHBG: [(testosterone × 100)/SHBG]. Serum inhibin B was analyzed by a double antibody enzyme-immunometric assay using a monoclonal antibody raised against the inhibin β_B-subunit in combination with labeled antibody raised against the α-subunit (Groome et al. 1996). The detection limit was 20 pg/mL, and intra- and interassay CVs were < 15% and < 18%, respectively. Ratios between hormones were calculated by simple division: LH/testosterone, LH/free testosterone, FSH/inhibin B.

For determination of phthalate monoesters, breast milk samples were thawed and placed in a water bath at 37°C to get a homogeneous sample without a separate fat layer. An aliquot of 3 mL was removed for liquid extraction using a mixture of ethyl acetate and cyclohexane (95:5) followed by a two-step solid phase extraction as described in detail previously (Mortensen et al. 2005). Determination of phthalate monoesters was accomplished by high-pressure liquid chromatography (Surveyor; Thermo Finnigan, San Jose, CA, USA) with a Betasil Phenyl column (100 × 2.1 mm × 3 µm) (Thermo Hypersil-Keystone, Thermo Finnigan). Column temperature was 25°C, injection volume was 20 µL, and flow rate was 350 µL/min. A Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer in combination with the Xcalibur software program was used for detection and quantitation (Thermo Electron Corporation, San Jose, CA, USA). The instrument was run in negative mode using the electro spray source (ESI). Detection limits were in the range of 0.01 to 0.5 µg/L. Recoveries at two different levels ranging from 2 to 120 µg/L were included using different milk samples and the CV (percent) was calculated from measurements of real duplicate determinations during the project period. Recovery was 93–104% and method variation was 5–15%. All analyses were carried out blinded with regard to the child's clinical examination or serum hormone concentration of reproductive hormones.

Statistics

Population characteristics are given as medians and percentiles (2.5th, 97.5th). Differences between boys with and without cryptorchidism were analyzed by Mann-Whitney *U*-test (Table 1). Six breast milk samples with undetectable values for mono-methyl phthalate (mMP) were assigned the limit of detection (LOD) value for mMP (0.01 µg/L) before statistical analysis. Estimates of daily exposure levels (micrograms per day) were calculated by the following equation: phthalate monoester concentration in breast milk (micrograms per liter) × infant weight at 3 months (kilograms) × average milk consumption (0.120 L/kg/day). To calculate the exposure as micrograms per kilograms per day, phthalate concentration (micrograms per liter) was multiplied by 0.120 L.

We tested differences in phthalate monoester concentration in breast milk and daily exposures between countries, as well as phthalate monoester concentration in breast milk between boys with and without cryptorchidism, by Mann-Whitney *U*-test. We tested associations between phthalate monoesters by Spearman correlations.

To investigate the relationship between hormone levels and phthalate monoesters, we used a multiple regression analysis with log-transformed data. Potential confounders (gestational age, weight for gestational age, parity, smoking, diabetes, country of origin) were investigated, and finally only country of origin was entered as confounder.

We then tested associations between six phthalate monoesters and seven reproductive hormones as well as three hormonal ratios with partial Spearman correlations while adjusting for country differences. Because of the small sample size, we obtained *p*-values for the exact distributions by Monte Carlo permutation.

Results

Table 1 describes the study population characteristics for boys with and without cryptorchidism separately, which do not show significant differences for maternal or infant parameters.

Concentrations of phthalate monoesters showed large interindividual variations, with single samples being extreme compared with the country median (Table 2). Except for mMP, all six phthalates were detectable in all breast milk samples; mMP could not be found in 2 of 65 (3%) Danish and in 4 of 65 (6%) Finnish samples. Mono-isononyl phthalate (miNP) showed the highest concentration of all phthalate monoesters. There was a significant difference between Denmark and Finland for four phthalate monoesters (Figure 1). Finland showed higher values for mono-*n*-butyl phthalate (mBP) ($p = 0.0001$), monobenzyl phthalate (mBzP) ($p = 0.0001$), and mEHP ($p = 0.001$), but lower values for miNP ($p = 0.056$). Individual phthalate monoester concentrations were positively correlated to each other ($r = 0.24$–0.43, $p = 0.0001$), except for miNP, which was not correlated to any other phthalate.

There was no significant difference ($p = 0.440$–0.823) between children with or without cryptorchidism with regard to any phthalate monoester concentration in breast milk, if analyzed either separately for each country (data not shown) or together. Median concentrations (cases vs. controls) were 0.094 versus 0.103 µg/L mMP; 0.898 versus 0.976 µg/L for mono-ethyl phthalate (mEP); 10.25 versus 9.09 µg/L for mBP; 1.25 versus 1.20 µg/L for mBzP; 10.55 versus 10.51 µg/L for mEHP; and 98.52 versus 91.75 µg/L for miNP.

Information on the use of mechanical breast pumps was available only for the Danish samples, in which significantly higher mEP and mBP levels were observed when breast pumps were used ($p = 0.001$ and $p = 0.02$, respectively). In the laboratory, we tested whether incubation of breast milk at 37°C for 2 hr in a commonly used polycarbonate breast pump influenced the level of phthalate monoesters measured. No increase or decrease in any of the six phthalate monoesters could be observed.

Phthalate monoesters were associated with hormones related to Leydig cell function. Both mEP and mBP showed significant positive correlations with SHBG (Table 3). A 10-fold increase in mEP/mBP raised serum SHBG levels by 15% (3–28%) and 8% (–1 to 18%), respectively. Both mBzP and miNP showed the same tendency but did not reach statistical significance. The LH:free testosterone ratio was significantly positively correlated to mMP, mEP, and mBP, with similar, nonsignificant tendencies for mEHP ($p < 0.095$) and miNP ($p < 0.099$). A 10-fold increase in mMP, mEP, and mBP concentrations raised the LH:free testosterone ratio by mean 19% (–3 to 46%), 26% (–1 to 60%), and 18% (–2 to 44%), respectively. Correlations between LH:testosterone ratio and mMP, mEP, mBP, and mEHP showed tendencies ($p < 0.10$) in the same direction (positive association), but none reached statistical significance. Free testosterone was significantly negatively correlated with mBP, with a change of –15% (–29 to +1%) over a 10-fold increase of mBP. Both mEP and mEHP showed similar, nonsignificant tendencies. Examples of regression plots for mEP are shown in Figure 2. miNP dose dependently increased serum LH and showed a tendency toward increasing total testosterone. A 10-fold increase of miNP raised LH levels by 97% (23–214%). We found similar correlations between phthalate monoester concentrations in breast milk and serum levels of reproductive hormones when analyzing only the group of boys without cryptorchidism from both countries (Table 4).

Findings concerning the two markers of Sertoli cell function (FSH, inhibin B) were subtle (Table 3). There was a tendency toward an increase in inhibin B with increasing concentration of mBzP and mEHP, which did not reach statistical significance. All phthalate monoesters showed a negative correlation to the FSH:inhibin B ratio, which reached statistical significance only for mEHP. In the control group, no associations were seen between markers of Sertoli cell function and phthalate monoester concentration in breast milk (Table 4). Parity, maternal smoking during pregnancy, diabetes, gestational age, and weight for gestational age were not significant confounders for the association between phthalate monoesters and reproductive hormones.

Estimates of average infant exposure to phthalate monoesters (micrograms per day and micrograms per kilogram per day) are given in Table 5. The lowest exposure was seen for mMP, mEP, and mBzP, and the highest was seen for mBP, mEHP, and miNP. There were significant country differences in daily intake of mBP and mBzP.

Table 1. Study population characteristics [median (2.5th–97.5th percentile)] and *p*-value for differences between boys with and without cryptorchidism (Mann-Whitney *U*-test).

Characteristic	Boys with cryptorchidism ($n = 62$)	Healthy boys ($n = 68$)	*p*-Value
No. (Denmark, Finland)	29, 33	36, 32	0.484
Maternal age (years)	29.7 (21.8–39.5)	29.3 (22.2–40.5)	0.415
Maternal diabetes (yes, no)	5, 57	1, 67	0.075
Maternal smoking (yes, no)	13, 49	13, 55	0.793
Parity			
1	37	47	0.230
2	14	13	
≥ 3	11	8	
Gestational age (days)	280 (236–296)	282 (227–296)	0.089
Weight for gestational age (%)	0.82 (–30.2 to 28.9)	0.15 (–22.4 to 33.7)	0.795
Birth weight (kg)	3.60 (1.99–4.76)	3.68 (2.78–4.81)	0.598
Birth length (cm)	52 (43–59)	52 (48–57)	0.284
Placenta weight (g)	550 (280–942)	600 (350–1,228)	0.164
Weight 3 months (kg)	6.55 (4.88–8.25)	6.58 (5.31–8.51)	0.804
Length 3 months (cm)	63 (60–67)	63 (57–67)	0.431

Table 2. Median concentration [range (µg/L)] of six phthalate monoesters in human breast milk samples 1997–2001, collected as additive aliquots from 1 to 3 months postnatally.

Phthalate	Denmark ($n = 65$)	Finland ($n = 65$)	*p*-Value	LOD (µg/L)	Detection rate (%)
mMP	0.10 (< 0.01–5.53)	0.09 (< 0.01–0.37)	0.355	0.01	95
mEP	0.93 (0.07–33.6)	0.97 (0.25–41.4)	0.976	0.01	100
mBP	4.3 (0.6–10,900)	12 (2.4–123)	0.0001	0.05	100
mBzP	0.9 (0.2–14)	1.3 (0.4–26)	0.0001	0.05	100
mEHP	9.5 (1.5–191)	13 (4.0–1,410)	0.001	0.10	100
miNP	101 (27–469)	89 (28–230)	0.056	0.50	100

Country differences were tested by Mann-Whitney *U*-test.

Discussion

We found subtle but significant dose-dependent associations between neonatal exposure to phthalate monoesters in breast milk and

levels of reproductive hormones in boys at 3 months of age. The most consistent findings were that higher phthalate monoester concentrations in mothers' breast milk were linked to higher serum SHBG levels and LH:free testosterone ratios. For mBP, higher exposure was also associated with lower free serum testosterone levels. Similar antiandrogen effects have been observed previously in newborn rodents exposed perinatally to phthalate diesters and monoesters (Albro et al. 1989; Foster et al. 2001; Gray et al. 2000; Jarfelt et al. 2005; Li et al. 1998). Average exposure of infants from breast milk was lower than doses used in animal exposure studies. However, exposure through lactation is only one of many potential exposure routes, and children are exposed to many phthalates simultaneously. Estimates for the tolerable daily intake (TDI; milligrams per kilogram per day) for phthalate diesters in humans are currently 0.05 for mMP, mBzP, and mEHP; 0.2 for di-ethyl phthalate (DEP); 0.1 for DBP; and 0.15 for DiNP (European Commission 2004; Kavlock et al. 2002a, 2002b; Petersen and Breindahl 2000). A direct comparison of exposure to monoesters to these values is not possible. The magnitude of the average exposure levels appears to be below currently established TDIs for the diesters. However, individual children can exceed these limits, especially for the metabolites of DBP and DEHP.

Our study showed that the absolute concentration of phthalate monoesters such as mBP, mBzP, and mEHP in breast milk differed between countries despite close geographic vicinity and comparable lifestyles. Thus, values reported here may not be directly applicable to other populations. Another large population survey has likewise shown that age, sex, and ethnicity affect concentrations of phthalate monoesters measured in urine (Silva et al. 2004b). In this study we also demonstrated that very little is known about individual sources of phthalate exposure and exposure variation and even less about potential differences in metabolism between people. A considerable intraindividual variation in urinary phthalate metabolite excretion has been demonstrated (Hauser et al. 2004). Only one previous study measured the same phthalate metabolites as in our study in three pooled samples of breast milk from American women. That study reported lower levels than observed here or previously in Danish control women (Calafat et al. 2004; Mortensen et al. 2005). Our LOD values were considerably lower than those in the American study, and we assessed the average phthalate exposure from 1 to 3 months postnatally, which may explain part of the differences. Our study showed, in agreement with the American report, that the metabolites of longer-chain phthalates such as mEHP and miNP, in particular, are found in milk samples, whereas the shorter-chain compounds such as mEP are more prevalent in urine and serum in the glucuronidated form (Silva et al. 2004a). This finding corresponds well to the increasing fat solubility of longer-chain phthalates, which may facilitate their higher segregation into milk. The detection rate of phthalate metabolites in human breast milk was 95% for mMP and 100% for all others including miNP, higher than in most other human matrices studied such as urine, serum, amniotic fluid, and saliva (Duty et al. 2005, Hauser et al. 2004; Silva et al. 2004a, 2004b, 2004c).

Breast milk samples can potentially be contaminated with phthalate diesters during collection and storage. It is generally accepted that phosphoric acid should be added immediately

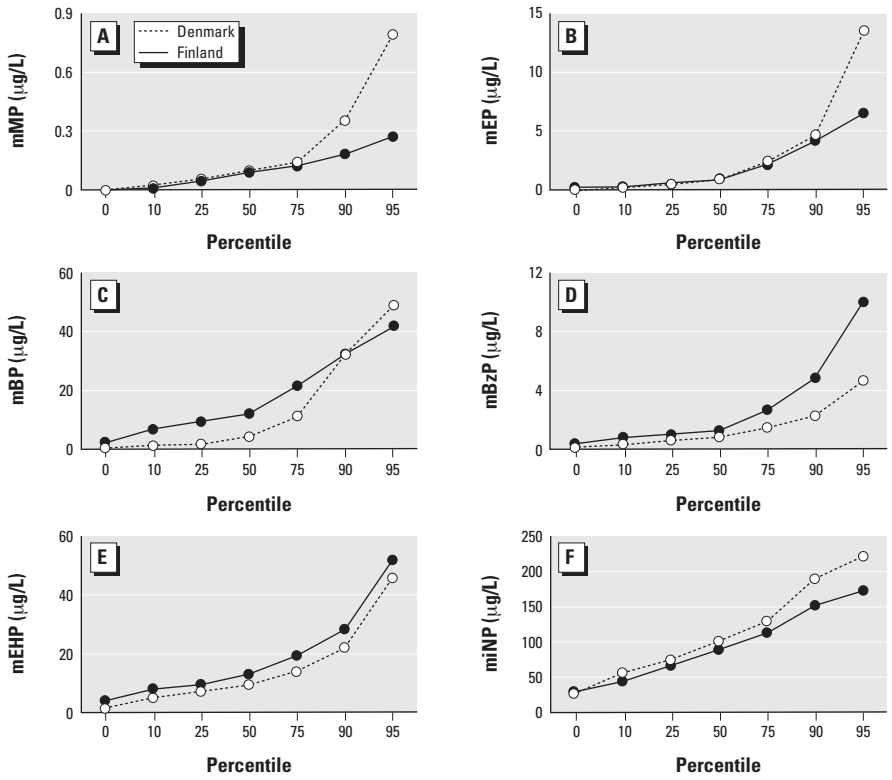


Figure 1. Concentration of six phthalate monoesters (μg/L) in human breast milk samples from Denmark (n = 65) and Finland (n = 65), 1997–2001, collected between 1 and 3 months postnatally as additive aliquots. Data are given as percentile distribution. (*A*) mMP, (*B*) mEP, (*C*) mBP, (*D*) mBzP, (*E*) mEHP, (*F*) miNP.

Table 3. Spearman correlations between concentrations of phthalate monoesters (μg/L) in human breast milk and reproductive hormones in serum of boys 3 months of age with and without cryptorchidism (n = 96).

Hormone	mMP	mEP	mBP	mBzP	mEHP	miNP
Leydig cell function						
SHBG (nmol/L)	0.076	0.323	0.272	0.188	0.080	0.187
p-Value	0.475	0.002	0.01	0.074	0.452	0.076
LH (IU/L)	0.159	0.185	0.076	0.049	0.001	0.243
p-Value	0.128	0.075	0.469	0.643	0.994	0.019
Testosterone (nmol/L)	0.009	–0.010	–0.040	0.115	–0.09	0.184
p-Value	0.929	0.927	0.705	0.271	0.392	0.078
Free testosterone	–0.065	–0.191	–0.220	–0.007	–0.169	0.070
p-Value	0.539	0.068	0.033	0.951	0.107	0.510
LH:testosterone ratio	0.174	0.189	0.200	–0.007	0.180	0.092
p-Value	0.098	0.072	0.056	0.946	0.087	0.384
LH:free testosterone ratio	0.210	0.323	0.282	0.060	0.175	0.174
p-Value	0.044	0.002	0.006	0.570	0.095	0.099
Sertoli cell function						
FSH (IU/L)	0.041	0.050	–0.083	0.045	–0.122	–0.043
p-Value	0.696	0.633	0.417	0.668	0.240	0.681
Inhibin B (pg/mL)	0.101	0.116	0.055	0.181	0.185	–0.004
p-Value	0.333	0.267	0.596	0.083	0.075	0.972
FSH:Inhibin B ratio	–0.006	–0.027	–0.132	–0.049	–0.204	–0.058
p-Value	0.951	0.796	0.202	0.641	0.050	0.584

p-Values are not adjusted for multiple testing.

to serum samples to inhibit esterase activity. We did not find any difference in phthalate monoester concentrations after thawing of the breast milk samples with and without the addition of phosphoric acid (Mortensen et al. 2005), showing that no contamination occurred during the analytic handling of the sample. However, another study reported a rapid increase in monoesters after spiking of defrosted breast milk samples with DEHP, DBP, and benzylbutylphthalate if no phosphoric acid was added (Calafat et al. 2004). Thus, we cannot exclude that contamination may occur during collection at home (e.g., from air particles, dust, locally applied cosmetics, or containers such as breast pumps), augmenting the concentration of monoesters before the samples reach the hospital. We decided against the addition of phosphoric acid because samples were collected at home as additive aliquots of unknown volume. Open handling of phosphoric acid was not considered safe. In addition, the final volume of the sample delivered at the 3-month examination could not be predicted, which inhibited the precise calculation of the necessary volume of phosphoric acid. However, if contamination of breast milk samples had occurred at random in our study, our chances of finding associations with endogenous hormones would have been considerably weakened. Thus, we believe that our findings of associations between phthalate monoester levels and reproductive hormones may be potentially underestimated, not the reverse. However, the absolute concentrations reported here must be interpreted with caution and may not be applicable to other study settings.

In rodents, secondary-step metabolites of DEHP such as mono(2-ethyl-5-hydroxyhexyl) phthalate (mEHHP) and mono(2-ethyl-5-oxyhexyl) phthalate (mEOHP) are suspected to be more toxic than DEHP or mEHP itself (Latini 2005). There are first reports on measurement of these metabolites in human matrices such as urine, serum, and saliva. However, these reports are seriously hampered by lack of analytic sensitivity, with the majority of samples being below the LOD or quantification (Calafat et al. 2004; Kato et al. 2004; Silva et al. 2005). Further research into the analytic method for determination of oxidative metabolites in breast milk and other matrices, as well as studies into their toxicity for humans, are urgently needed.

To our knowledge, this is the first report showing an association between phthalate exposure and reproductive hormones in boys. We are aware of problems in connection with conducting multiple analyses. Additional analysis of the data including only healthy boys without cryptorchidism showed comparable findings with the total group, thus strengthening our conclusion of a subtle effect on endogenous hormone levels related to Leydig cell function. We did not find any correlation of phthalate exposure with cryptorchidism, which disagrees with rodent studies (Imajima et al. 2001; Jarfelt et al. 2005; Kavlock et al. 2002a, 2002b). However, we did not find significant differences in birth weight, weight for gestational age, or gestational age between boys with and without cryptorchidism, which is in contrast to our findings in the total baby cohort from which this data set is derived (Boisen et al. 2004). This indicates that our study groups may be too small to detect subtle changes related to the presence or absence of congenital cryptorchidism. As testicular descent occurs prenatally, our postnatal exposure assessment during lactation may have missed the critical window for development. We have no data currently on how milk contamination with phthalate metabolites compares with prenatal exposure through placenta and amniotic fluid. Increased SHBG is an indirect sign of reduced androgen activity (Belgorosky and Rivarola 1985). Elevated LH levels, together with decreased free testosterone and elevated LH:free testosterone ratio, are consistent with an adverse effect on Leydig cell function leading to a reduced biologic androgen effect.

Physiologically, there is negative feedback between testosterone levels in serum and pituitary LH secretion. In addition, miNP was associated with serum LH levels. This finding is of particular concern, as DiNP today has replaced DEHP in many applications such as food packaging, flexible plastic toys, and flooring, and the exposure levels found in our study were the highest among all six phthalate monoesters analyzed. Our findings concerning mEHP, a phthalate with a higher reproductive toxicity in animal experiments than its parent compound, DEHP, also showed a tendency toward an antiandrogenic effect, which did not reach statistical significance. This may be related to the limited number of samples in our study, the extreme variation of individual exposure levels, or species differences.

Interestingly, in an independent, parallel American study of another mother–child cohort, women with highest excretion of mEP, mBP, mBzP, and mono-isobutyl phthalate (miBP) in urine during pregnancy gave birth to boys who were less virilized, as judged from smaller than expected measurements of anogenital distance. We did not measure anogenital distance in our cohort. However, our observations on the associations between mBP and mEP and markers of Leydig cell function, in particular, are consistent with the American study (Swan et al. 2005) in terms of an antiandrogenic effect of phthalate exposure in infants boys, assessed by two different biomarkers. We also found an effect of mEP on SHBG levels and on the ratio between LH and free testosterone, whereas rodent studies did not show any toxicity of its parent compound, DEP (Gray et al. 2000). Because mEP was also one of four phthalate metabolites affecting anogenital distance in the American baby study, these observations may indicate a species difference in vulnerability that will have to be studied thoroughly in the future.

We observed hormonal changes indicating an effect on Sertoli cells—an increase in inhibin B—which we did not expect and therefore

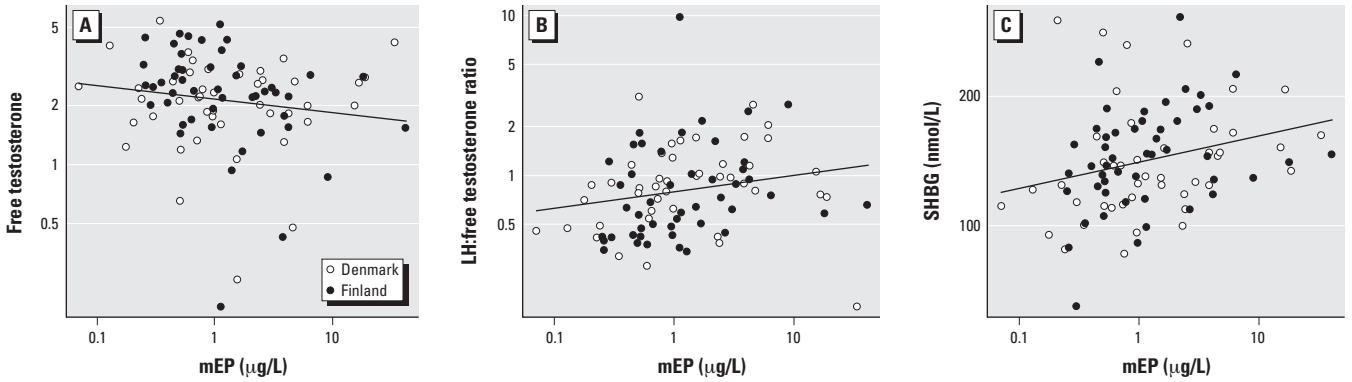


Figure 2. Regression plots of mEP levels (μg/L) in human breast milk and serum hormonal levels in boys 3 months of age (n = 96). The x- and y-axes for mEP, free testosterone, SHBG, and LH:free testosterone ratio are logarithmic. The slopes (confidence interval) of the regression lines are (*A*) free testosterone, 0.86 (0.69–1.06); (*B*) LH:free testosterone ratio, 1.26 (0.99–1.60); and (*C*) SHBG 1.15 (1.03–1.28). Example of interpretation: A 10-fold increase in mEP, for example, from 1 to 10 μg/L, is associated with a 15% increase in SHBG from 140.5 to 161.3 nmol/L.

considered a random finding. However, a recent report found a similar, equally weak, effect in adult men (Duty et al. 2005). We cannot yet explain this observation and, unfortunately, inhibin B levels have not been measured in animal experiments. However, histologic studies of rodent testes exposed pre- and perinatally show vacuolization of Sertoli cells (Borch et al. 2004, 2005) and apoptosis of spermatogenic cells in cultures of mouse seminiferous tubules (Suominen et al. 2003).

Although the hormonal changes in the boys were linked to phthalate monoester levels in breast milk, we cannot exclude that fetal exposure may be a contributing factor to altered postnatal hormone levels. Levels of phthalate monoesters in breast milk may be a proxy of general maternal exposure: the women with high levels of phthalate monoesters in milk may also be among those with highest exposures during pregnancy. Phthalates can cross the placenta; DEHP and mEHP have been detected in maternal and cord blood (Latini et al. 2003) and their metabolites were found in amniotic fluid (Silva et al. 2004c). Sources of phthalate exposures in women can be inhalation (Adibi et al. 2003), contamination via building materials and furniture, use of consumer products including cosmetics (Koo and Lee 2004), and food items (Anderson et al. 2001). Thus, exposure to some phthalates such as DEHP and DiNP is likely to be constant rather than episodic, whereas others such as DMP and DEP, through their presence in cosmetics, are more influenced by personal habits. The observed effects on endocrine hormone levels could therefore be late effects of fetal exposure or additive fetal and neonatal exposure through the mother, or exposure to a home environment generally rich in phthalates during pregnancy and infancy.

We observed a significant difference in mEP and mBP levels depending on whether breast milk was sampled with or without use of a breast pump. This observation is unlikely to have any link to the observed associations between phthalate monoester levels in breast milk and infant hormone levels. Danish and Finnish mothers have several months of maternity leave, and breast pumps are not used regularly for infant feeding. Furthermore, in our laboratory we could not observe any leaching of phthalates from a commonly used mechanical breast pump into breast milk. Thus, it remains to be verified whether our observed difference in mEP and mBP was a true effect of breast pump use or a chance finding.

Individuals will often be exposed to a mixture of endocrine-disrupting chemicals—for example, phthalates in cosmetics usually coexist with parabens, which also act as endocrine disruptors. In situations where mixtures of agents even in minute concentrations contribute to the adverse effects (Silva et al. 2002), causal relationships are extremely difficult to establish. We cannot rule out that our findings could be due to one or multiple unknown factors, the presence of which is associated with the use of phthalates. Breast feeding has numerous benefits for infant nutrition and for establishing an ideal mother–child relationship. We do not believe that our data should be taken to argue against breast-feeding, because effects on reproductive hormones were subtle. In addition, phthalates have also been found in other major nutrition sources for infants (Latini et al. 2004; Mortensen et al. 2005; Petersen and Breindahl 2000; Shea 2003).

In conclusion, our findings support the hypothesis that the human testis may be vulnerable to phthalate exposure during development. Before any regulatory action is considered, further studies on health effects of phthalate esters and their metabolites in humans are urgently needed. These studies should be aimed specifically at verifying or refuting our findings. In this respect, breast milk samples may be a valuable biologic matrix for assessing long-term average exposure, not only to persistent toxicants but also to endocrine disrupters with a short half-life such as phthalates. In addition, the postnatal activation of the pituitary–gonadal axis in infants appears to be a valuable biomarker for early detection of endocrine disruption in humans.

Table 4. Spearman correlations between concentrations of phthalate monoesters (μg/L) in human breast milk and reproductive hormones in serum of boys 3 months of age without cryptorchidism (n = 46).

Hormone	mMP	mEP	mBP	mBzP	mEHP	miNP
Leydig cell function						
SHBG (nmol/L)	0.128	0.449	0.296	0.252	0.134	0.069
p-Value	0.410	0.003	0.050	0.107	0.388	0.662
LH (IU/L)	0.419	0.322	0.082	0.053	0.156	0.273
p-Value	0.006	0.037	0.611	0.733	0.319	0.078
Testosterone (nmol/L)	0.082	–0.027	–0.219	0.031	–0.076	–0.062
p-Value	0.594	0.860	0.152	0.840	0.623	0.689
Free testosterone	–0.028	–0.301	–0.427	–0.169	–0.205	–0.109
p-Value	0.861	0.053	0.004	0.283	0.190	0.493
LH:testosterone ratio	0.302	0.344	0.386	0.094	0.357	0.323
p-Value	0.047	0.023	0.008	0.547	0.018	0.034
LH:free testosterone ratio	0.389	0.517	0.462	0.169	0.371	0.319
p-Value	0.010	0.0005	0.001	0.283	0.014	0.038
Sertoli cell function						
FSH (IU/L)	0.060	0.112	–0.084	0.060	–0.106	–0.152
p-Value	0.630	0.473	0.588	0.700	0.494	0.328
Inhibin B (pg/mL)	–0.003	–0.124	–0.173	–0.070	–0.017	–0.039
p-Value	0.982	0.414	0.211	0.650	0.908	0.800
FSH:Inhibin B ratio	–0.04	0.120	–0.104	0.029	–0.147	–0.108
p-Value	0.794	0.436	0.494	0.851	0.341	0.489

p-Values are not adjusted for multiple testing.

Table 5. Estimated individual intake (μg/day and μg/kg/day) of phthalate monoesters from breast milk given as medians (minimum–maximum).

	μg/day			μg/kg/day	
	Denmark	Finland	*p*-Value	Denmark	Finland
mMP	0.08 (< 0.01–3.92)	0.07 (< 0.01–0.27)	0.219	0.012 (< 0.01–0.66)	0.011 (< 0.01–0.04)
mEP	0.78 (0.06–22.7)	0.82 (0.18–31.0)	0.851	0.111 (0.01–4.03)	0.115 (0.03–4.97)
mBP	3.46 (0.45–7,550)	9.77 (1.95–92.2)	0.0001	0.517 (0.07–1,310)	1.450 (0.28–14.8)
mBzP	0.70 (0.14–10.1)	1.13 (0.38–19.8)	0.0001	0.104 (0.02–1.71)	0.169 (0.06–3.17)
mEHP	7.68 (0.92–153)	10.06 (3.0–904)	0.002	1.14 (0.18–23)	1.56 (0.47–169)
miNP	83.14 (19.7–332)	72.47 (22.0–194)	0.075	12.17 (3.20–56.3)	10.97 (3.40–27.6)

Breast milk samples were collected 1997–2001 in Denmark (n = 64) and Finland (n = 65). Country differences were tested by Mann-Whitney *U*-test.

Correction

In Figure 1D of the original manuscript published online, the data for Finland and Denmark were reversed; they have been corrected here.

References

Adibi JJ, Perera FP, Jedrychowski W, Camann DE, Barr D, Jacek R, et al. 2003. Prenatal exposure to phthalates among women in New York City and Krakow, Poland. Environ Health Perspect 111:1719–1722.

Albro PW, Chapin RE, Corbett JT, Schroeder J, Phelps JL. 1989. Mono-2-ethylhexyl phthalate, a metabolite of di-(2-ethylhexyl) phthalate, causally linked to testicular atrophy in rats. Toxicol Appl Pharmacol 100:193–200.

Anderson WA, Castle L, Scotter MJ, Massey RC, Springall C. 2001. A biomarker approach to measuring human dietary exposure to certain phthalate diesters. Food Addit Contam 18:1068–1074.

Andersson A-M, Toppari J, Haavisto A-M, Petersen JH, Simell T, Simell O, et al. 1998. Longitudinal reproductive hormone profiles in infants: peak of inhibin B levels in infant boys exceeds levels in adult men. J Clin Endocrinol Metab 83:675–681.

Belgorosky A, Rivarola MA. 1985. Sex hormone binding globulin

response to testosterone. An androgen sensitivity test. Acta Endocrinol 1985:130–138.

Boisen KA, Kaleva M, Main KM, Virtanen HE, Haavisto A-M, Schmidt IM, et al. 2004. Difference in prevalence of congenital cryptorchidism in infants between two Nordic countries. Lancet 363:1264–1269.

Borch J, Dalgaard M, Ladefoged O. 2005. Early testicular effects in rats perinatally exposed to DEHP in combination with DEHA—apoptosis assessment and immunohistochemical studies. Reprod Toxicol 19:517–525.

Borch J, Ladefoged O, Hass U, Vinggaard AM. 2004. Steroidogenesis in fetal male rats is reduced by DEHP and DINP, but endocrine effects of DEHP are not modulated by DEHA in fetal, prepubertal and adult male rats. Reprod Toxicol 18:53–61.

Calafat AM, Slakman AR, Silva MJ, Herbert AR, Needham LL. 2004. Automated solid phase extraction and quantitative analysis of human milk for 13 phthalate metabolites. J Chromatogr B Analyt Technol Biomed Life Sci 805:49–56.

Duty SM, Calafat AM, Silva MJ, Ryan L, Hauser R. 2005. Phthalate exposure and reproductive hormones in adult men. Hum Reprod 20:604–610.

European Commission. 2004. Guidelines of the Scientific Committee on Food. Brussels:European Commission. Available: http://europa.eu.int/comm/food/fs/sc/scf/out82_en.pdf [accessed 6 January 2006].

Fisher JS, Macpherson S, Marchetti N, Sharpe RM. 2003. Human "testicular dysgenesis syndrome": a possible model using in-utero exposure of the rat to dibutyl phthalate. Hum Reprod 18:1383–1394.

Foster PM, Mylchreest E, Gaido KW, Sar M. 2001. Effects of phthalate esters on the developing reproductive tract of male rats. Hum Reprod Update 7:231–235.

Gray LE Jr, Ostby J, Furr J, Price M, Veeramachaneni DN, Parks L. 2000. Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. Toxicol Sci 58:350–365.

Groome NP, Illingworth PJ, O'Brien M, Pai R, Rodger FE, Mather JP, et al. 1996. Measurement of dimeric inhibin B throughout the human menstrual cycle. J Clin Endocrinol Metab 81:1401–1405.

Hauser R, Meeker JD, Park S, Silva MJ, Calafat AM. 2004. Temporal variability of urinary phthalate metabolite levels in men of reproductive age. Environ Health Perspect 112:1734–1740.

Imajima T, Shono T, Kai H, Zakaria O, Suita S. 2001. The biological effect of phthalate esters on transabdominal migration of the testis in fetal rats in comparison with the antiandrogen flutamide. Pediatr Surg Int 17:164–166.

Jarfelt K, Dalgaard M, Hass U, Borch J, Jacobsen H, Ladefoged O. 2005. Antiandrogenic effects in male rats perinatally exposed to a mixture of di(2-ethylhexyl) phthalate and di(2-ethylhexyl) adipate. Reprod Toxicol 19:505–515.

Kato K, Silva MJ, Reidy JA, Hurtz III D, Malek NA, Needham LL, et al. 2004. Mono(2-ethyl-5-hydroxyhexyl) phthalate and mono(2-ethyl-5-oxyhexyl) phthalate as biomarkers for human exposure assessment to di-(2-ethylhexyl) phthalate. Environ Health Perspect 112:327–330.

Kavlock R, Boekelheide K, Chapin R, Cunningham M, Faustman EM, Foster P, et al. 2002a. NTP Center for the Evaluation of Risks to Human Reproduction: phthalates expert panel report on the reproductive and developmental toxicity of di-*n*-butyl phthalate. Reprod Toxicol 16:489–527.

Kavlock R, Boekelheide K, Chapin R, Cunningham M, Faustman EM, Foster P, et al. 2002b. NTP Center for the Evaluation of Risks to Human Reproduction: phthalates expert panel report on the reproductive and developmental toxicity of di-*iso*-nonyl phthalate. Reprod Toxicol 16:679–708.

Koo HJ, Lee BM. 2004. Estimated exposure to phthalates in cosmetics and risk assessment. J Toxicol Environ Health A 67:1901–1914.

Latini G. 2005. Monitoring phthalate exposure in humans. Clin Chim Acta 361(1–2):20–29.

Latini G, DeFelice C, Presta G, DelVecchio A, Paris I, Ruggieri F, et al. 2003. Exposure to di(2-ethylhexyl)phthalate in humans during pregnancy. Biol Neonate 83:22–24.

Latini G, DeFelice C, Verrotti A. 2004. Plasticizers, infant nutrition and reproductive health. Reprod Toxicol 19:27–33.

Li L-H, Jester WF, Orth JM. 1998. Effects of relatively low levels of mono-(2-ethylhexyl) phthalate on cocultured Sertoli cells and gonocytes from neonatal rats. Toxicol Appl Pharmacol 153:258–265.

Main KM, Smith IM, Skakkebæk NE. 2000. A possible role for reproductive hormones in newborn boys: progressive hypogonadism without the postnatal testosterone peak. J Clin Endocrinol Metab 85:4905–4907.

Marsál K, Persson PH, Larsen T, Lilja H, Selbing A, Sultan B. 1996. Intrauterine growth curves based on ultrasonically estimated fetal weight. Acta Paediatr 85:843–848.

Mortensen GK, Main KM, Andersson A-M, Leffers H, Skakkebæk NE. 2005. Determination of phthalate monoesters in human breast milk, consumer milk and infant formula by tandem mass spectrometry (LC/MS/MS). Anal Bioanal Chem 382:1084–1092.

Petersen JH, Breindahl T. 2000. Plasticizers in total diet samples, baby food and infant formula. Food Addit Contam 17:133–141.

Shea KM. 2003. Pediatric exposure and potential toxicity of phthalate plasticizers. Pediatrics 111:1467–1474.

Silva E, Rajapakse N, Kortenkamp A. 2002. Something from "nothing"—eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. Environ Sci Technol 15:1751–1756.

Silva MJ, Barr DB, Reidy JA, Kato K, Malen NA, Hodge CC, et al. 2004a. Glucuronidation patterns of common urinary and serum monoester phthalate metabolites. Arch Toxicol 77:561–567.

Silva MJ, Barr DB, Reidy JA, Malek NA, Hodge CC, Caudill SP, et al. 2004b. Urinary levels of seven phthalate metabolites in the U.S. population from the National Health and Nutrition Examination Survey (NHANES) 1999–2000. Environ Health Perspect 112:331–338.

Silva MJ, Reidy JA, Herbert AR, Preau JL Jr, Needham LL, Calafat AM. 2004c. Detection of phthalate metabolites in human amniotic fluid. Bull Environ Contam Toxicol 72:1226–1231.

Silva MJ, Reidy JA, Samandar E, Herbert AR, Needham LL, Calafat AM. 2005. Detection of phthalate metabolites in human saliva. Arch Toxicol 79(11):647–652.

Suominen JS, Linderborg J, Nikula H, Hakovirta H, Parvinen M, Toppari J. 2003. The effects of mono-2-ethylhexyl phthalate, adriamycin and *N*-ethyl-*N*-nitrosourea on stage-specific apoptosis and DNA synthesis in the mouse spermatogenesis. Toxicol Lett 143:163–173.

Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, et al. 2005. Anogenital distance—a marker of fetal androgen action—is decreased in male infants following phthalate exposure during pregnancy. Environ Health Perspect 113:1056–1061.

World Medical Association. 2004. World Medical Association Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects. Available: http://www.wma.net/e/policy/b3.htm [accessed 6 January 2006].

The Relationship between Environmental Exposures to Phthalates and DNA Damage in Human Sperm Using the Neutral Comet Assay

Susan M. Duty,[1] Narendra P. Singh,[2] Manori J. Silva,[3] Dana B. Barr,[3] John W. Brock,[3] Louise Ryan,[4] Robert F. Herrick,[1] David C. Christiani,[1,5] and Russ Hauser[1]

[1]Department of Environmental Health, Occupational Health Program, Harvard School of Public Health, Boston, Massachusetts, USA; [2]Department of Bioengineering, University of Washington, Seattle, Washington, USA; [3]National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, Georgia, USA; [4]Department of Biostatistics, Harvard School of Public Health, and Department of Biostatistical Science, Dana-Farber Cancer Institute, Boston, Massachusetts, USA; [5]Department of Medicine, Harvard Medical School, Boston, Massachusetts, USA

Phthalates are industrial chemicals widely used in many commercial applications. The general population is exposed to phthalates through consumer products as well as through diet and medical treatments. To determine whether environmental levels of phthalates are associated with altered DNA integrity in human sperm, we selected a population without identified sources of exposure to phthalates. One hundred sixty-eight subjects recruited from the Massachusetts General Hospital Andrology Laboratory provided a semen and a urine sample. Eight phthalate metabolites were measured in urine by using high-performance liquid chromatography and tandem mass spectrometry; data were corrected for urine dilution by adjusting for specific gravity. The neutral single-cell microgel electrophoresis assay (comet assay) was used to measure DNA integrity in sperm. VisComet image analysis software was used to measure comet extent, a measure of total comet length (micrometers); percent DNA in tail (tail%), a measure of the proportion of total DNA present in the comet tail; and tail distributed moment (TDM), an integrated measure of length and intensity (micrometers). For an interquartile range increase in specific gravity–adjusted monoethyl phthalate (MEP) level, the comet extent increased significantly by 3.6 μm [95% confidence interval (95% CI), 0.74–6.47]; the TDM also increased 1.2 μm (95% CI, –0.05 to 2.38) but was of borderline significance. Monobutyl, monobenzyl, monomethyl, and mono-2-ethylhexyl phthalates were not significantly associated with comet assay parameters. In conclusion, this study represents the first human data to demonstrate that urinary MEP, at environmental levels, is associated with increased DNA damage in sperm. *Key words:* **comet assay, DNA damage, environmental, human sperm, phthalates, urinary metabolites.** *Environ Health Perspect* **111:1164–1169 (2003). doi:10.1289/ehp.5756 available via *http://dx.doi.org/* [Online 6 December 2002]**

Phthalates are multifunctional chemicals used to hold color and scent in consumer and personal care products (Koo et al. 2002); as carpet backing and as solvents in paints, glue, and insect repellents (ATSDR 1999); and to soften a wide range of plastic goods (Bradbury 1996). Di(2-ethylhexyl) phthalate (DEHP), one of the more commonly used phthalates, leaches from blood products, intravenous and dialysate bags, and tubing made with polyvinyl chloride (Nässberger et al. 1987). Phthalates are also present in drinking water, air, and food (ATSDR 1995, 1999, 2000). Despite the rapid metabolism and elimination of most phthalates (Koo et al. 2002; Nässberger et al. 1987; Peck and Albro 1982), theoretically a constant steady state may be reached because of chronic and repetitive, low-level exposures from dietary ingestion and from many commonly used products.

Evidence of widespread exposure of the U.S. population to phthalates comes from two recent studies on the levels of phthalate metabolites in urine samples collected for the Third National Health and Nutrition Examination Survey (NHANES III) (Blount et al. 2000b) and NHANES 1999 (CDC 2001). The NHANES surveys collect biological samples and information about the health and diet of people in the United States (National Center for Health Statistics 2001). Four phthalate metabolites—monoethyl phthalate (MEP), mono-2-ethylhexyl phthalate (MEHP), mono-*n*-butyl phthalate (MBP), and monobenzyl phthalate (MBzP)—were present in more than 75% of U.S. subjects sampled (Blount et al. 2000b; CDC 2001).

Evidence of general population exposure to phthalates (Blount et al. 2000b; CDC 2001), as well as *in vitro* studies suggesting that some phthalates are hormonally active (Harris et al. 1997; Nakai et al. 1999) and animal studies showing associations between some phthalates and testicular toxicity (Gangolli 1982; Li et al. 1998; Parks et al. 2000; Sharpe et al. 1995; Thomas et al. 1982), has generated both public and scientific concern about potential reproductive effects of phthalates. Recent *in vitro* studies using the alkaline comet assay (single-cell gel electrophoresis) found di-*n*-butyl phthalate (DBP) and di-isobutyl phthalate (DiBP) to be genotoxic in human epithelial cells of the upper aerodigestive tract (Kleinsasser et al. 2000a), as well as in mucosal cells and lymphocytes (Kleinsasser et al. 2000b). Additionally, the comet assay was used to detect DNA damage in human lymphocytes induced by *in vitro* exposure to DEHP and MEHP (Anderson et al. 1999).

A lack of consensus on which semen quality tests are the best predictors of human male fertility has led to the development of several new methods to evaluate semen quality. The traditional semen analysis measures sperm concentration, motility, and morphology (World Health Organization 1999). Several laboratory techniques are used to evaluate sperm DNA, such as the sperm chromatin structure assay (SCSA) (Evenson et al. 1991). The SCSA may prove to be a useful clinical test because of its high repeatability and its ability to measure an aspect of fertility that differs from what can be offered by the traditional semen analysis (Evenson et al. 1999). Other DNA tests include fluorescence *in situ* hybridization, used to measure aneuploidy, as well as assays used to measure DNA integrity, including single-cell microgel electrophoresis (comet assay) and the terminal deoxynucleotidyl transferase–mediated dUTP-biotin end-labeling (TUNEL) assay (Lähdetie et al. 1996; Martin 1993; Sun et al. 1997; World Health Organization 1999).

Few published human studies have examined the effect of environmental chemicals on DNA integrity in sperm as measured by the comet assay. In the present study, we used the neutral comet assay to measure DNA integrity in human sperm and investigated whether DNA integrity was associated with urinary concentrations of five phthalate monoesters.

Materials and Methods

Subjects. The study was approved by the Harvard School of Public Health and Massachusetts General Hospital (MGH) Human Subjects Committee, and all subjects signed an informed consent form. Subjects were recruited from an ongoing semen quality

Address correspondence to R. Hauser, Dept. of Environmental Health, Occupational Health Program, Bldg. 1 Rm. 1405, 665 Huntington Ave., Boston, MA 02115-9957 USA. Telephone: (617) 432-3326. Fax: (617) 432-0219. E-mail: rhauser@hohp.harvard.edu

We also thank A. Calafat, Z. Chen, S. Swan, J. Frelich, L. Godfrey-Bailey, A. Larson, N. Mercedat, L. Pothier, A. Trisini, P. Hengel, and Impuls computergestützte Bildanalyse GmbH, Germany.

We acknowledge the National Institute of Environmental Health Sciences grants ES09718, ES00002. NIH training grant T32 ES07069 supported S.M.D.

The authors declare they have no conflict of interest.

Received 6 May 2002; accepted 4 December 2002.

study and are male partners of a subfertile couple who presented to the MGH Andrology Laboratory (Boston, MA) between January 2000 and October 2001 for semen analysis as part of an infertility investigation. Eligible men were those between 20 and 54 years old. Men presenting for postvasectomy semen analysis were excluded.

Semen sample collection. Semen was produced on site at MGH by masturbation into a sterile plastic specimen cup after a recommended period of abstinence of 48 hr. After liquefaction at 37°C for 30 min, pH, color, and viscosity measurements were made, and semen was analyzed. Sperm concentration and motility were measured by computer-aided sperm analysis (version 10HTM-IVOS; Hamilton-Thorn, Beverly, MA) using manufacturer instructions, and morphology was measured manually using Kruger strict criteria. Remaining raw semen was then frozen in 0.25-mL cryogenic straws (CryoBiosystem, I.M.V. Division, San Diego, CA) by immersing the straws directly into liquid nitrogen (–196°C). Previous work in our laboratory showed that this freezing method produced results that were highly correlated with results from fresh, unfrozen samples (Duty et al. 2002). The straws were thawed by gently shaking in a 37°C water bath for 10 sec, and the semen was immediately processed for comet assay.

Comet assay. The entire procedure was conducted under low indirect incandescent light (60 W) to minimize light-induced damage to sperm DNA. All chemicals were purchased from VWR Scientific (West Chester, PA) unless otherwise specified. After thawing, semen (with approximately 2×10^5 sperm) was mixed with 400 µL 0.7% agarose (3:1 high resolution; Amresco, Solon, OH). Fifty microliters of this semen/agarose mixture was embedded between two additional 200-µL layers of 0.7% agarose on specially designed, partially frosted, microgel electrophoresis glass slides with a clear central window (Erie Scientific, Portsmouth, NH). Cover glasses were removed before submersion of slides in a cold lysing solution (4°C) of 2.5 M NaCl, 100 mM EDTA tetrasodium salt, 10 mM Tris-base (pH 10), 1% sodium lauroyl sarcosine, and 1% Triton X-100 (Roche Diagnostics Corp., Indianapolis, IN); this step mainly dissolves the cell membrane to make chromatin accessible for the next two enzyme digestion steps. The slides were then transferred to enzyme treatment (2.5 M NaCl, 5 mM Tris, 0.05% sodium lauroyl sarcosine with pH adjusted to 7.4), and 10 mg/mL of RNase (Amresco, Solon, OH). After 4 hr at 37°C, the slides were transferred into enzyme treatment plus 1 mg/mL DNase-free proteinase K (Amresco, Solon, OH) for 18 hr at 37°C. These two steps are crucial for decondensing sperm chromatin and allowing migration of broken DNA out of the nucleus. Slides were then equilibrated in neutral electrophoresis solution (300 mM sodium acetate, 100 mM Tris, pH 9) for 20 min before being electrophoresed under neutral conditions at 12 V and 130 mA for 1 hr at room temperature. This was followed by precipitation and fixation of cells first in absolute alcohol mixed with 10 M ammonium acetate for 15 min, and then in 70% ethanol with 100 mg of spermine for 30 min. The resulting slides were air dried and then stained with YOYO dye (Molecular Probes, Eugene, OR), an intensely fluorescent DNA dye. Fluorescent comet patterns were examined with a Leica fluorescence microscope model DMLB under 400× magnification and fluoroisothiocyanate filter combination.

Image analysis. VisComet image analysis software, kindly donated by Impuls Computergestützte Bildanalyse GmbH (Gilching, Germany) was used to measure comet extent, percent DNA in tail (tail%), and tail distributed moment (TDM) on 100 sperm in each semen sample. Comet extent is a measure of total comet length from the beginning of the head to the last visible pixel in the tail. This measurement is similar to that obtained by manual analysis using an eyepiece micrometer. Tail% is a measurement of the proportion of the total DNA that is present in the tail. The TDM is an integrated value that takes into account both the distance and intensity of comet fragments. The formula used to calculate the TDM is

$$M_{\text{dist}} = \Sigma\,(I^{*}X) \,/\, \Sigma\, I,$$

where $\Sigma\, I$ is the sum of all intensity values that belong to the head, body, or tail, and X is the x-position of intensity value. In addition to these two parameters, cells too long to measure with VisComet (> 300 µm; "long cells") were tallied and used as a third measure of DNA damage. Because of the presence of long cells in most subjects, more than 100 cells may have been screened and scored to allow for the measurement of comet extent, tail%, and TDM on 100 cells per subject.

Urinary phthalate metabolites. We measured the monoester phthalate metabolites because of potential sample contamination from the ubiquitous parent diester and because the metabolites are believed to be the active toxicant, not the parent diester compounds (Li et al. 1998; Peck and Albro 1982). Eight urinary phthalate metabolites—MEP, monomethyl phthalate (MMP), MEHP, MBP, MBzP, mono-*n*-octyl phthalate (MOP), mono-3-methyl-5-dimethylhexyl (isononyl) phthalate (MINP), and monocyclohexyl phthalate (MCHP)—were measured in a single spot urine sample, collected in a sterile specimen cup on the same day as the semen sample. Because more than 75% of the study population had levels of MCHP, MINP, and MOP below the limit of detection (LOD), the results for these metabolites were not informative and are not included in the analysis. The analytical approach has been described in detail elsewhere (Blount et al. 2000a). Briefly, urinary phthalate metabolite determination involved enzymatic deconjugation of metabolites from the glucuronidated form, solid-phase extraction, separation with high-performance liquid chromatography, and detection by tandem mass spectrometry. Detection limits were in the low nanogram per milliliter range. Reagent blanks and $^{13}C_4$-labeled internal standards were used along with conjugated internal standards to increase precision of measurements. One method blank, two quality control samples (human urine spiked with phthalates), and two standards were analyzed along with every 10 unknown urine samples (Blount et al. 2000a). Analysts were blind to all information concerning subjects.

Specific gravity adjustment. We measured urinary specific gravity to identify unreliable urine samples and to normalize phthalate levels for differences in urinary dilution between subjects. We used a hand-held refractometer (National Instrument Company, Inc., Baltimore, MD) that was calibrated with deionized water before each batch of measurements. Phthalate concentrations were corrected for specific gravity by the formula

$$P_c = P[(1.024 - 1)/(\text{SG} - 1)],$$

where P_c is the specific gravity–corrected phthalate concentration (nanograms per milliliter), P is the observed phthalate concentration (nanograms per milliliter), and SG is the specific gravity of sample (Boeniger et al. 1993; Teass et al. 1998). Specific gravity–adjusted phthalate levels were used in statistical modeling as a continuous predictor variable without transformation.

Statistical analysis. For data analysis, we used Statistical Analysis Software (SAS), version 8.1 (SAS Institute Inc., Cary, NC), and we performed descriptive analyses of subject characteristics. In separate univariate and multiple regression analyses, the mean of 100 cells per person was used for each of the dependent variables: comet extent, tail%, and TDM. Because mean comet extent and TDM were normally distributed (Shapiro-Wilk test p-values > 0.35), they were used untransformed in the regression analyses. However, because tail% was not normally distributed, analyses using both untransformed and log-transformed tail% were performed. Because the results and their interpretation did not differ, we chose to present only the untransformed tail% results for ease of interpretation. We used regression analysis to explore the relationship between the comet parameters and specific gravity–adjusted

urinary phthalate metabolite levels, adjusting for covariates. Covariates for inclusion were based on statistical and biologic considerations (Hosmer and Lemeshow 1989). Because the number of long cells in a semen sample was not normally distributed, it was transformed using the arcsine transformation (Zar 1984) and regressed on urinary phthalates. Spearman correlation coefficients were used to determine correlations among phthalate monoesters and among comet parameters.

In the regression models, age was modeled as a continuous independent variable after checking for appropriateness using a quadratic term. Abstinence time was modeled as an ordinal five-category variable (2 or fewer days, 3, 4, 5, and 6 or more days), and smoking status was used as a dummy variable (current and former vs. never). Race was categorized into four groups: white, African American, Hispanic, and other.

Results

Of the 253 men recruited into an ongoing semen quality study, 1 dropped out and 168 subjects had both phthalate levels and comet analysis results (Figure 1). Because the study initially did not archive semen for future comet assay analyses, the first 46 subjects recruited were excluded even though their urine had been collected and analyzed for phthalates. An additional 17 subjects were excluded from the data analysis because they could not provide a urine sample at the time of semen collection, and 12 subjects with archived semen samples had no sperm (azoospermic), and so the comet assay could not be performed. Nine samples were lost when cryostraws exploded upon thawing.

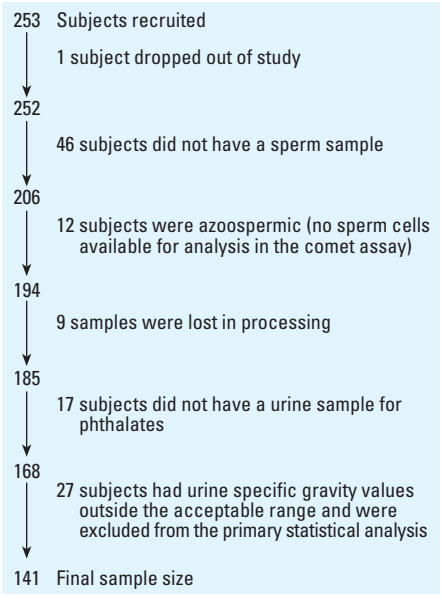


Figure 1. Subject exclusions. Of the 253 subjects recruited to participate, 168 subjects had semen and urine samples available for analysis. The final sample size for statistical analysis was 141 subjects; 27 subjects were excluded because urine specific gravity was out of the acceptable range (< 1.010 or > 1.030).

Demographic information and semen parameters are given in Table 1. The mean (± SD) of age and body mass index of the 168 subjects was 36.3 ± 5.7 years and 28.2 ± 4.6 years, respectively. About 77% of subjects were white, 7.8% African American, 7.2% Hispanic, and 7.8% other. Most subjects (72.0%) never smoked, and only 9.5% were current smokers (smoked within the past month). The mean (± SD) semen concentration, motility, and strict morphology were 111.1 ± 91.0 million/mL, 52.4 ± 23.6% motile sperm, and 7.1 ± 4.5% normally shaped sperm, respectively. Although the mean values are all larger than the reference values for each semen parameter [World Health Organization (WHO) 1999], 52% of subjects had values for one or more semen parameters below the WHO reference values. Twenty-four subjects (14.3%) had < 20 million sperm/mL, 68 subjects (40.5%) had < 50% motile sperm, and 38 subjects (22.6%) had < 4% normally shaped sperm. Eighty-one subjects (48%) had semen parameters that were above the WHO reference values for all three semen parameters.

The distribution of comet parameters and specific gravity–adjusted urinary phthalate metabolite levels are shown in Table 2. Of the 168 subjects with both comet assay results and urinary phthalate monoester levels, 27 were excluded from the primary data analysis because specific gravity values were outside the acceptable range (< 1.010 or > 1.030) (Boeniger et al. 1993; Teass et al. 1998). MEP was detected in

Table 1. Subject demographics and semen and comet parameters (n = 168).

Parameters	Mean ± SD	No. (%)
Age	36.3 ± 5.7	
Body mass index	28.2 ± 4.6	
Race[a]		
White		129 (77.2)
African American		13 (7.8)
Hispanic		12 (7.2)
Other		13 (7.8)
Smoking status		
Never smoker		121 (72.0)
Ever smoker		47 (28.0)
Current smoker		16 (9.5)
Ex-smoker		31 (18.5)
Semen parameters		
Sperm concentration (million/mL)	111.1 ± 91.0	
Subjects < 20 million sperm/mL		24 (14.3)
Sperm motility (% motile)	52.4 ± 23.6	
Subjects < 50% motile sperm		68 (40.5)
Sperm morphology[b] (% normal morphology)	7.1 ± 4.5	
Subjects < 4% normal morphology		38 (22.6)
Comet assay parameters[c]		
Comet extent (μm)	125.3 ± 32.3	
Tail%	20.9 ± 7.7	
TDM (μm)	59.0 ± 13.7	
Number of long cells	10.0 ± 12.0	
Subjects without long cells		17 (10.1)
Subjects with long cells		151 (89.9)

[a]One person missing race. [b]Kruger strict criteria used for morphology determination. [c]VisComet image analysis software was used to measure comet extent (microns), tail%, and TDM. One person missing both TDM and tail%.

Table 2. Distribution of urinary phthalate levels[a] and comet assay parameters[b].

			Percentile						
	No.[c]	Minimum	5th	25th	50th	75th	95th	Maximum	Geometric mean
Phthalate monoester[d]									
MEP	141	9.8	27.6	59.6	160.5	503	2029.8	5396.2	186.8
MBzP	141	< LOD	1.5	3.9	8.9	15.6	39.8	138.3	7.8
MBP	141	< LOD	3.9	10.7	18.1	31.0	69.9	2623.4	18.2
MEHP	141	< LOD	< LOD	1.9	5.2	13.8	86.0	319.3	7.1
MMP	141	< LOD	< LOD	2.1	4.9	11.4	32.1	104.7	6.1
Comet parameters									
Comet extent	141	53.4	72.8	105.7	124.8	149.8	180.0	219.2	121.5
Tail%	140	9.9	12.8	16.6	19.8	22.5	35.1	61.6	19.9
TDM	140	29.5	37.4	48.8	58.8	68.7	82.6	91.2	57.4
Number of long cells	141	0	0	2	6	13	31	73	6.8

[a]Specific gravity–adjusted urinary phthalate monoester concentration (ng/mL urine). [b]VisComet image analysis software was used to measure comet extent, tail%, and TDM. [c]Twenty-seven of 168 subjects were excluded because specific gravity value was out of acceptable range (< 1.010 or > 1.030), and one subject was missing TDM and tail% measures. [d]LODs for phthalates (ng/mL) are as follows: MEP, 1.0; MBzP, 0.8; MBP, 0.6; MEHP, 1.2; MMP, 0.71.

100% of subjects, MBP and MBzP in at least 95% of subjects, and MEHP and MMP in at least 75% of subjects. The phthalate monoester with the highest concentration was MEP, ranging from 9.8 to 5396.2 (ng/mL) ppb with a geometric mean of 186.8 ppb. The median MEP concentration ranged from 9- to 32-fold higher than any other phthalate metabolite. Interquartile ranges (IQRs) varied considerably among the phthalates, from 443 ppb for MEP to only 9.3 ppb for MMP. The IQR of comet parameters also varied, 44 μm for comet extent and 20 μm for TDM. Fifty percent of comet extents were between 105 and 150 μm, with < 5% of cells longer than 180 μm or shorter than 70 μm. Figure 2A–C demonstrates the heterogeneity of comet tail lengths within an individual; Figure 2D depicts the comet cell referred to as a "long cell," a cell that was too long to measure with image analysis software.

The mean (± SD) comet extent, tail%, and TDM were 125.3 ± 32.3 μm, 20.9 ± 7.7%, and 59.0 ± 13.7 μm, respectively. Comet extent ranged from 53.4 to 219.2 μm, tail% from 9.9 to 61.6%, and TDM from 29.5 to 91.2 μm. The number of long cells in a semen sample ranged from 0 to 73 cells. We counted the number of long cells in addition to the 100 cells measured with the VisComet software per sample. Comet extent and TDM were highly correlated ($r = 0.90$, $p < 0.0001$); however, tail% was moderately correlated with comet extent ($r = 0.35$, $p < 0.0001$) and weakly correlated with TDM ($r = 0.14$; $p = 0.10$). Moderate correlations existed between the number of long cells and both comet extent and TDM ($r = 0.45$ and $r = 0.44$, respectively; $p < 0.0001$), but the correlation between long cells and tail% was weak ($r = 0.10$, $p = 0.26$). The five phthalate monoesters were only weakly or moderately correlated with each other. The strongest correlation was found between MBP and MBzP ($r = 0.43$; $p < 0.001$), which is expected because the diester butyl benzyl phthalate (BBzP) gives rise to both MBP and MBzP in a 5:3 ratio (NTP 2000). The weakest correlation was found between MMP and MBzP ($r = 0.015$, $p = 0.9$), suggesting that exposures to these phthalates may come from different sources.

In the univariate linear regression analyses, although not statistically significant, comet extent and TDM were longer in current smokers than in never smokers (127.7 μm vs. 125.5 μm, and 63.0 μm vs. 59.3 μm, respectively). Tail% was higher in former smokers but lower in current smokers compared with never smokers (23.5% and 18.4% vs. 20.1%, respectively; $p = 0.03$ and 0.44, respectively). The relationships between age and both comet extent and TDM were inconsistent and not significant. Comet extent increased 0.007 μm/year [95% confidence interval (CI), –0.92 to 1.06], but TDM decreased 0.14 μm/year (95% CI, –0.56 to 0.27). Tail% significantly increased 0.22%/year (95% CI, 0.00 to 0.44). The number of long cells increased marginally as age increased (< 1 cell/year, $p = 0.07$), but it was not associated with smoking. In contrast to the unstable relationships between age and smoking with comet assay parameters, MEP was significantly associated with comet extent; the regression coefficient was 3.5 μm/IQR (95% CI, 0.73 to 6.33). Tail% was not significantly associated with MEP (–0.11%/IQR; 95% CI, –0.78 to 0.56). The relationship between TDM and MEP and MBzP was less stable and failed to reach statistical significance, with increases of 1.10 μm/IQR (95% CI, –0.10 to 2.30) and 1.18 μm/IQR (95% CI, –0.25 to 2.62), respectively. There were no significant, or even suggestive, univariate relationships between specific gravity–adjusted phthalate levels and the number of long cells. The regression coefficients were close to zero, and the confidence intervals were wide.

Although the relationships between smoking and comet assay parameters were inconsistent, we included smoking as a potential confounder in the multiple regression models because several studies have reported increased DNA damage in smokers (Fraga et al. 1996; Sun et al. 1997; Ündeğer et al. 1999). Additionally, age was included in the multiple regression models because there is evidence that DNA damage increases with age (Møller et al. 2000; Singh et al. 2001). Generally, the crude and adjusted coefficients in the multiple regression models were similar, indicating that there was minimal confounding by age and smoking status.

The final multiple regression models are summarized in Table 3. After adjusting for age and smoking status, for an IQR increase in specific gravity–adjusted MEP concentrations, the comet extent significantly increased 3.61 μm (95% CI, 0.74 to 6.47), whereas TDM increased 1.17 μm but was of borderline statistical significance (95% CI, –0.05 to 2.38). Tail% decreased marginally with an IQR change in MEP, although it was not significant (–0.17%/IQR; 95% CI, –0.81 to 0.47). In contrast, the coefficients for the relationships between MBP and MEHP and comet extent, tail%, and TDM were near zero and not significant. In addition, the coefficients for the adjusted relationships between phthalate levels and the number of long cells were close to zero and nonsignificant (data not shown).

In a sensitivity analysis, we reanalyzed the data after including the 27 subjects that were excluded from the primary analysis because their urine specific gravity was outside the acceptable range. In the reanalysis, the coefficients for the relationships between MEP and comet extent and TDM, adjusted for age and smoking, were statistically significant and became larger, 3.67 μm/IQR (95% CI, 1.07 to 6.26) and 1.23 μm/IQR (95% CI, 0.12 to 2.34), respectively. The relationship between MEP and tail% remained essentially unchanged (–0.09%/IQR; 95% CI, –0.66 to 0.48). The

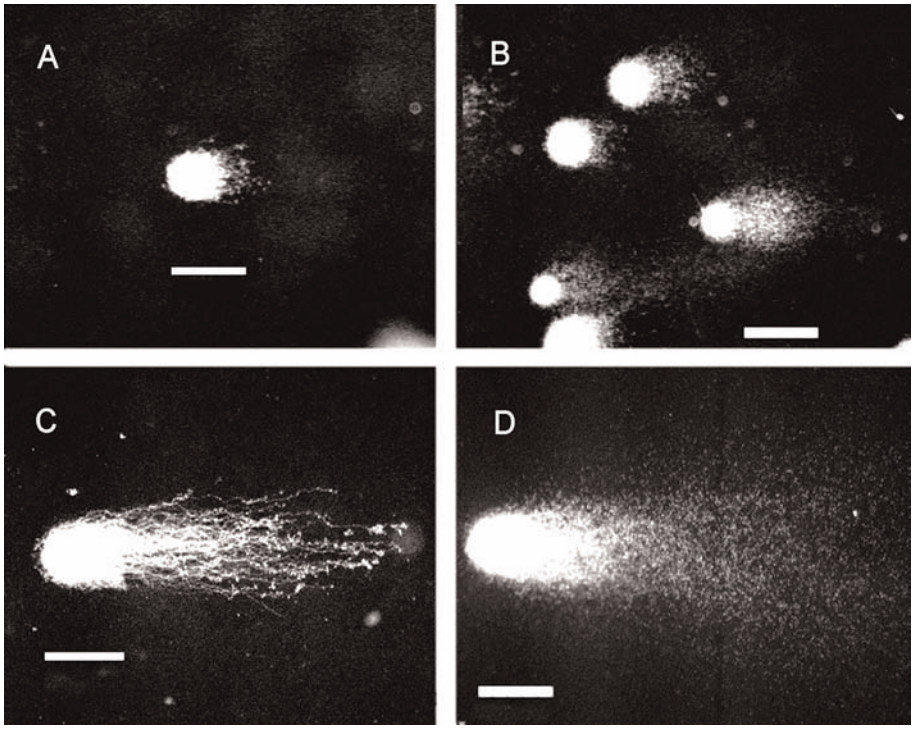


Figure 2. Comet tail length. (*A*) Cell with a short comet tail. (*B*) Considerable heterogeneity of comet tail lengths within an individual. (*C*) Cell with a long comet tail. (*D*) Comet referred to as a "long cell," a cell with highly fragmented DNA that was too long to measure with image analysis software. Bars = 50 μm.

regression coefficients for the relationship between MBzP and comet extent and TDM increased moderately in magnitude, to 2.89 μm/IQR (95% CI, –0.10 to 5.87) for comet extent and to 1.20 μm/IQR (95% CI –0.07 to 2.46) for TDM. The coefficients and confidence intervals for MBP and MEHP were similar to the results of the initial analysis, and their interpretation remained unchanged. The coefficients for MMP and comet extent and TDM became smaller in magnitude, although the confidence intervals narrowed.

Discussion

The present study represents one of the first human studies to report an association between urinary levels of MEP, at levels found in the general population, and increased DNA migration in sperm, assessed using the neutral comet assay. Specifically, there was a statistically significant positive association between urinary MEP and mean comet extent and a suggestive association with TDM. However, no significant associations were found between comet assay parameters and other urinary phthalate metabolites, including MBP, MBzP, MEHP, and MMP.

Animal data suggest that several phthalates, including butyl benzyl phthalate (BBzP), DBP, DEHP, and MEHP, are associated with damage to the testes and decreased sperm production (Gangolli 1982; Li et al. 1998; Parks et al. 2000; Sharpe et al. 1995; Thomas et al. 1982); however, there are only a few studies on the genotoxicity of these agents. Using the alkaline comet assay, researchers have found evidence of genotoxicity with *in vitro* studies examining lymphocytes and mucosal cells of the upper aerodigestive tract after exposure to DBP and DiBP (Kleinsasser et al. 2000a, 2000b, 2001). In another study using the alkaline comet assay on human leukocytes, an association between MEHP and DEHP and increased tail moments was found (Anderson et al. 1999). In contrast to those studies, in the present study we found no linear association between MEHP or MBP and sperm DNA migration. It is unclear whether the different results derive from the different cell types studied or the use of the neutral assay in the present study, compared with the use of the alkaline assay in the other studies.

In the neutral comet assay, a cell with fragmented DNA has the appearance of a "comet" with a brightly fluorescent head and a fluorescent tail whose intensity represents the relative amount of DNA strand breaks present (Hughes et al. 1997; Singh and Stephens 1998; Singh et al. 1988). The comet assay for human sperm was adapted from methods used on somatic cells, which can be conducted under alkaline or neutral conditions. Neutral conditions were used for human sperm because of the abundance of alkali-sensitive sites in sperm. Alkaline test conditions can induce damage at alkali-labile sites and produce DNA strand breaks (Singh et al. 1989).

In previous studies using the comet assay, changes in DNA migration (comet length) were detected at low levels of x-irradiation, 12.5 centigrays (rads) in human lymphocytes (Singh and Stephens 1997) and 50 centigrays (rads) in human sperm (Duty et al. 2002). Therefore, we considered comet extent and TDM to represent sensitive quantitative measures of DNA damage. However, tail moment is purported to be a more sensitive measure of DNA damage than TDM and comet extent. This increased sensitivity results from observations that with increasing levels of DNA damage, the tail length may not but tail% may continue to increase (McKelvey-Martin et al. 1993). In addition to these traditional comet assay parameters, we also tallied the number of long cells. We hypothesize that the long cell parameter represents an independent measure of DNA damage. This was partially confirmed by the weak correlation with the traditional comet assay parameters. Long cells represent very highly damaged cells. Definitive characterizations of the comet assay parameters and the significance of the long cells remain to be resolved. Although the present study was not designed to investigate this, we felt it was important to quantify long cells as a separate measure because this may prove useful in future studies using the neutral comet assay.

Although the data in the present study suggest an association between MEP and increased DNA migration in the comet assay, they must be interpreted cautiously because the phthalate levels are based on a single urine sample from a limited number of subjects. A recent study documents good reproducibility of urinary phthalate monoester measurements from day to day (Pearson correlation coefficients ranged from 0.5 to 0.8); however, this was in a small number of subjects (n = 46), all of whom were women and African American (Hoppin et al. 2002). Because phthalates have short half-lives (Nässberger et al. 1987; Peck and Albro 1982), spot urine samples reflect recent exposure. However, if a steady state of exposure and biologic burden is achieved with chronic repeated exposures to phthalates through the diet and the use of household and personal care products, then the utility of a single specimen is improved.

Urinary phthalate levels were normalized for urine dilution differences by adjusting for specific gravity. There are several methods to adjust for urine volume (Boeniger et al. 1993; Teass et al. 1998), and although creatinine is a frequently used form of adjustment, it is not always appropriate. If a compound is excreted primarily by tubular secretion, it is not appropriate to adjust for creatinine level (Teass et al. 1998). Although the methods of excretion of the phthalate monoesters measured in this study are unknown, terephthalic acid, a dicarboxylic acid phthalate analog, was found to be actively secreted by renal tubules and actively reabsorbed by the kidney (Tremaine and Quebbemann 1985). Furthermore, because organic compounds that are conjugated with glucuronides in the liver, such as phthalates, are eliminated by active tubular secretion (Boeniger et al. 1993), creatinine adjustment may not be appropriate. Additionally, creatinine levels may be confounded by muscularity, physical activity, urine flow, time of day, diet, and disease states (Boeniger et al. 1993; Teass et al. 1998). For these reasons, specific gravity was used to normalize phthalate levels. We excluded samples with specific gravity less than 1.010 or greater than 1.030 (Teass et al. 1998).

Phthalate levels in the present study were compared with levels measured in NHANES III (Blount et al. 2000b) and NHANES 1999 (CDC 2001). Even after limiting the NHANES III data to only men (Barr D. Personal communication; unpublished data), the phthalate levels were on average two to three times higher than those in the present study. The NHANES 1999 phthalate metabolite levels were also twice as high as those in our study. The two exceptions were MEP, which was similar between studies, and MEHP, which was twice as high in our study. MMP was not measured in NHANES data. It is unclear why MEHP levels were high in the present study because few subjects reported recent medical interventions including intravenous infusions, transfusions, or hemodialysis, which might account for higher MEHP levels. Despite the fact that the levels of phthalate

Table 3. Adjusted regression coefficients for a change in comet assay parameters associated with an IQR increase in phthalate monoester levels[a] (n^b = 141).

		Coefficients[c] for comet assay parameters[d]		
Phthalate monoester	IQR (ng/mL)	Comet extent (95% CI)	Tail% (95% CI)	TDM (95% CI)
MEP	443	3.61 (0.74 to 6.47)*	–0.17 (–0.81 to 0.47)	1.17 (–0.05 to 2.38)
MBzP	11.7	2.45 (–1.07 to 5.97)	0.05 (–0.07 to 0.82)	1.06 (–0.42 to 2.54)
MBP	20.3	–0.31 (–0.80 to 0.19)	–0.02 (–0.13 to 0.09)	–0.12 (–0.32 to 0.09)
MEHP	11.9	–0.19 (–1.54 to 1.16)	–0.01 (–0.30 to 0.29)	0.08 (–0.48 to 0.65)
MMP	9.3	2.20 (–1.51 to 5.90)	–0.12 (–1.24 to 0.40)	0.93 (–0.62 to 2.49)

[a]Specific gravity–adjusted urinary phthalate monoester concentration (ng/mL urine). [b]27 of 168 subjects were excluded because specific gravity value was out of acceptable range (< 1.010 or > 1.030). [c]Adjusted for age (continuous), and smoking (current, former versus never). [d]VisComet image analysis software was used to measure comet parameters. Coefficient units are (parameter)/IQR. *p = 0.015.

monoesters differed between our study and both NHANES studies, the metabolites with the highest levels were similar across studies (Blount et al. 2000b; CDC 2001). In all three studies, the highest phthalate levels were for MEP, followed by MBP and then MBzP.

Although the men in the present study may not be representative of men in Massachusetts, generalizability may not necessarily be limited. It is a misconception that generalization from a study group depends on the study group's being a representative subgroup of the target population (Rothman and Greenland 1998). For generalizability to be limited, the relationship between comet parameters and phthalates in this clinic population would need to differ from the relationship in the population being generalized to. We would need to speculate that men in this andrology clinic differ by some factor that alters their response to phthalates. Currently, there is no reason to suspect that men who visit this andrology clinic are more or less "sensitive" to phthalates than men who visit other clinics or men from the general population. However, until the results of the present study are replicated in larger and different populations, the generalizability of our results will remain unclear.

In summary, although a significant association was seen between MEP and one measure of DNA integrity in sperm, these results need to be duplicated in a larger study. The lack of significant associations between comet assay parameters and the other four phthalate metabolites may indicate a true difference in genotoxicity between monoesters. It may also reflect markedly different exposure distributions of these monoesters when compared with the broad exposure distribution of MEP. Conversely, the comet assay associations found with MEP may reflect conducting multiple comparisons. In conclusion, this is the first epidemiologic study to explore the association between urinary monoester phthalates at general population levels and DNA integrity in sperm. In addition, the present study demonstrates that the neutral comet assay is a potentially useful tool for detecting DNA damage in human sperm in epidemiologic studies.

References

Anderson D, Yu TW, Hinçcal F. 1999. Effect of some phthalate esters in human cells in the comet assay. Teratog Carcinog Mutagen 19:275–280.

ATSDR. 1995. Toxicological Profile for Diethyl Phthalate. Atlanta, GA:Agency for Toxic Substances and Disease Registry. Available: http://www.atsdr.cdc.gov/toxprofiles/ [accessed 28 March 2002].

———. 1999. Di-*n*-butyl phthalate. Atlanta, GA:Agency for Toxic Substances and Disease Registry. Available: http://www.atsdr.cdc.gov/toxprofiles/ [accessed 28 March 2002].

———. 2000. Toxicological Profile for Di(2-ethylhexyl) Phthalate. Atlanta, GA:Agency for Toxic Substances and Disease Registry. Available: http://www.atsdr.cdc.gov/toxprofiles/ [accessed 28 March 2002].

Blount BC, Milgram KE, Silva MJ, Malek NA, Reidy JA, Needham LL, et al. 2000a. Quantitative detection of eight phthalate metabolites in human urine using HPLC-APCI-MS/MS. Anal Chem 72:4127–4134.

Blount BC, Silva MJ, Caudill SP, Needham LL, Pirkle JL, Sampson EJ, et al. 2000b. Levels of seven urinary phthalate metabolites in a human reference population. Environ Health Perspect 108:979–982.

Boeniger MF, Lowry LK, Rosenberg J. 1993. Interpretation of urine results used to assess chemical exposure with emphasis on creatinine adjustments: a review. Am Ind Hyg Assoc J 54:615–627.

Bradbury J. 1996. UK panics over phthalates in babymilk formulae. Lancet 347:1541.

CDC. 2001. National Report on Human Exposure to Environmental Chemicals. Atlanta, GA:Centers for Disease Control and Prevention. Available: http://www.cdc.gov/nceh/dls/report 2001:1–6 [accessed 30 March 2001].

Duty SM, Singh NP, Ryan L, Chen Z, Lewis C, Huang T, et al. 2002. Reliability of the comet assay in cryopreserved human sperm. Hum Reprod 17:1274–1280.

Evenson DP, Jost LK, Baer RK, Turner TW, Schrader SM. 1991. Individuality of DNA denaturation patterns in human sperm as measured by the sperm chromatin structure assay. Reprod Toxicol 5:115–125.

Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, et al. 1999. Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in human fertility clinic. Hum Reprod 14:1039–1049.

Fraga CG, Motchnik PA, Wyrobek AJ, Rempel DM, Ames BN. 1996. Smoking and low antioxidant levels increase oxidative damage to sperm DNA. Mutat Res 351:199–203.

Gangolli SD. 1982. Testicular effects of phthalate esters. Environ Health Perspect 45:77–84.

Harris CA, Henttu P, Parker MG, Sumpter JP. 1997. The estrogenic activity of phthalate esters *in vitro*. Environ Health Perspect 105:802–811.

Hoppin JA, Brock JW, Davis BJ, Baird DD. 2002. Reproducibility of urinary phthalate metabolites in first morning urine samples. Environ Health Perspect 110:515–518.

Hosmer DW Jr, Lemeshow S. 1989. Model building strategies and methods for logistic regression. In: Applied Logistic Regression. New York:John Wiley & Sons, 82–134.

Hughes CM, Lewis SEM, McKelvey-Martin VJ, Thompson W. 1997. Reproducibility of human sperm DNA measurements using the alkaline single cell gel electrophoresis assay. Mutat Res 374:261–268.

Kleinsasser NH, Kastenbauer ER, Weissacher H, Muenzenrieder RK, Harréus UA. 2000a. Phthalates demonstrate genotoxicity on human mucosa of the upper aerodigestive tract. Environ Mol Mutagen 35:9–12.

Kleinsasser NH, Wallner BC, Kastenbauer ER, Weissacher H, Harréus UA. 2001. Genotoxicity of di-butyl-phthalate and di-iso-butyl-phthalate in human lymphocytes and mucosal cells. Teratog Carcinog Mutagen 21:189–196.

Kleinsasser NH, Weissacher H, Kastenbauer ER, Dirschedl P, Wallner BC, Harréus UA. 2000b. Altered genotoxicity in mucosal cells of head and neck cancer patients due to environmental pollutants. Eur Arch Otorhinolaryngol 257:337–342.

Koo J-W, Parham F, Kohn MC, Masten SA, Brock JW, Needham LL, et al. 2002. The association between biomarker-based exposure estimates for phthalates and demographic factors in a human reference population. Environ Health Perspect 110:405–410.

Lähdetie J, Ajosenpää-Saari M, Mykkänen J. 1996. Detection of aneuploidy in human spermatozoa of normal semen donors by fluorescence in situ hybridization. Environ Health Perspect 104:620–632.

Li L-H, Jester WF, Orth JM. 1998. Effects of relatively low levels of mono-(2-ethylhexyl) phthalate on cocultured Sertoli cells and gonocytes from neonatal rats. Toxicol Appl Pharmacol 153:258–265.

Martin RH. 1993. Detection of genetic damage in human sperm. Reprod Toxicol 7:47–52.

McKelvey-Martin VJ, Green MHL, Schmezer P, Pool-Zobel BL, De Méo MP, Collins A. 1993. The single cell gel electrophoresis assay (comet assay): a European review. Mutat Res 288:47–63.

Møller P, Knudsen LE, Loft S, Wallin H. 2000. The comet assay as a rapid test in biomonitoring occupational exposure to DNA-damaging agents and effect of confounding factors. Cancer Epidemiol Biomark Prev 9:1005–1015.

Nakai M, Tabira Y, Asai D, Yakabe Y, Shimyozu T, Noguchi M, et al. 1999. Binding characteristics of dialkyl phthalates for the estrogen receptor. Biochem Biophys Res Commun 254:311–314.

Nässberger L, Arbin A, Östelius J. 1987. Exposure of patients to phthalates from polyvinyl chloride tubes and bags during dialysis. Nephron 45:286–290.

National Center for Health Statistics. 2001. National Health and Nutrition Examination Survey. Hyattsville, MD:Centers for Disease Control and Prevention, National Center for Health Statistics, Division of Data Services. Available: http://www.cdc.gov/nchs/nhanes.htm [accessed 4 December 2001].

NTP. 2000. NTP-CERHR Expert Panel Report on Butyl Benzyl Phthalate. Research Triangle Park, NC:National Toxicology Program, Center for the Evaluation of Risks to Human Reproduction, 1–37.

Parks LG, Ostby JS, Lambright CR, Abbott BD, Klinefelter GR, Barlow NJ, et al. 2000. The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. Toxicol Sci 58:339–349.

Peck CC, Albro PW. 1982. Toxic potential for the plasticizer di(2-ethylhexyl) phthalate in the context of its disposition and metabolism in primates and man. Environ Health Perspect 45:11–17.

Rothman KJ, Greenland S. 1998. Precision and validity in epidemiologic studies. In: Modern Epidemiology (Rothman KJ, Greenland S, eds). 2nd ed. Philadelphia:Lippincott Williams & Wilkins, 133–134.

Sharpe RM, Fisher JS, Millar MM, Jobling S, Sumpter JP. 1995. Gestational and lactational exposure of rats to xenoestrogens results in reduced testicular size and sperm production. Environ Health Perspect 103:1136–1143.

Singh NP, Danner DB, Tice RR, McCoy MT, Collins GD, Schneider EL. 1989. Abundant alkali-sensitive sites in DNA of human and mouse sperm. Exp Cell Res 184:461–470.

Singh NP, McCoy MT, Tice RR, Schneider EL. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175:184–191.

Singh NP, Ogburn CE, Wolf N, van Belle G, Martin GM. 2001. DNA double-strand breaks in mouse kidney cells with age. Biogerontology 2:261–270.

Singh NP, Stephens RE. 1997. Microgel electrophoresis: sensitivity, mechanisms, and DNA electrostretching. Mutat Res 383:167–175.

———. 1998. X-ray-induced DNA double-strand breaks in human sperm. Mutagenesis 13:75–79.

Sun J-G, Jurisicova A, Casper RF. 1997. Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. Biol Reprod 56:602–607.

Teass AW, Biagini RE, DeBord G, Hull RD. 1998. Application of biological monitoring methods. In: NIOSH Manual of Analytical Method (Eller PM, ed). Cincinnati, OH:National Institute for Occupational Safety and Health, Division of Physical Sciences and Engineering, 52–62.

Thomas JA, Curto KA, Thomas MJ. 1982. MEHP/DEHP: Gonadal toxicity and effects on rodent accessory sex organs. Environ Health Perspect 45:85–88.

Tremaine LM, Quebbemann AJ. 1985. The renal handling of terephthalic acid. Toxicol Appl Pharmacol 77:165–174.

Ündeğer Ü, Zorlu AF, Başaran N. 1999. Use of the alkaline comet assay to monitor DNA damage in technicians exposed to low-dose radiation. J Occup Environ Med 41:693–698.

World Health Organization. 1999. WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction. New York:Cambridge University Press.

Zar JH. 1984. The arcsine transformation. In: Biostatistical Analysis. Englewood Cliffs, NJ:Prentice-Hall, Inc., 239–241.

TOXICOLOGICAL SCIENCES **58,** 350–365 (2000)

Perinatal Exposure to the Phthalates DEHP, BBP, and DINP, but Not DEP, DMP, or DOTP, Alters Sexual Differentiation of the Male Rat

L. Earl Gray Jr.,*,[1] Joseph Ostby,* Johnathan Furr,* Matthew Price,* D. N. Rao Veeramachaneni,† and Louise Parks*

*Endocrinology Branch, MD 72, U.S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Reproductive Toxicology Division, Research Triangle Park, North Carolina 27711; and †Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, Colorado 80523-1683

Received May 26, 2000; accepted July 24, 2000

In mammals, exposure to antiandrogenic chemicals during sexual differentiation can produce malformations of the reproductive tract. Perinatal administration of AR antagonists like vinclozolin and procymidone or chemicals like di(2-ethylhexyl) phthalate (DEHP) that inhibit fetal testicular testosterone production demasculinize the males such that they display reduced anogenital distance (AGD), retained nipples, cleft phallus with hypospadias, undescended testes, a vaginal pouch, epididymal agenesis, and small to absent sex accessory glands as adults. In addition to DEHP, di-n-butyl (DBP) also has been shown to display antiandrogenic activity and induce malformations in male rats. In the current investigation, we examined several phthalate esters to determine if they altered sexual differentiation in an antiandrogenic manner. We hypothesized that the phthalate esters that altered testis function in the pubertal male rat would also alter testis function in the fetal male and produce malformations of androgen-dependent tissues. In this regard, we expected that benzyl butyl (BBP) and diethylhexyl (DEHP) phthalate would alter sexual differentiation, while dioctyl tere- (DOTP or DEHT), diethyl (DEP), and dimethyl (DMP) phthalate would not. We expected that the phthalate mixture diisononyl phthalate (DINP) would be weakly active due to the presence of some phthalates with a 6-7 ester group. DEHP, BBP, DINP, DEP, DMP, or DOTP were administered orally to the dam at 0.75 g/kg from gestational day (GD) 14 to postnatal day (PND) 3. None of the treatments induced overt maternal toxicity or reduced litter sizes. While only DEHP treatment reduced maternal weight gain during the entire dosing period by about 15 g, both DEHP and DINP reduced pregnancy weight gain to GD 21 by 24 g and 14 g, respectively. DEHP and BBP treatments reduced pup weight at birth (15%). Male (but not female) pups from the DEHP and BBP groups displayed shortened AGDs (about 30%) and reduced testis weights (about 35%). As infants, males in the DEHP, BBP, and DINP groups displayed femalelike areolas/nipples (87, 70, and 22% ($p <$ 0.01), respectively, versus 0% in other groups). All three of the phthalate treatments that induced areolas also induced a significant incidence of reproductive malformations. The percentages of males with malformations were 82% ($p < 0.0001$) for DEHP, 84% ($p < 0.0001$) for BBP, and 7.7% ($p < 0.04$) in the DINP group. In summary, DEHP, BBP, and DINP all altered sexual differentiation, whereas DOTP, DEP, and DMP were ineffective at this dose. Whereas DEHP and BBP were of equivalent potency, DINP was about an order of magnitude less active.

Key Words: phthalates; DINP; DEHP; BBP; DOTP; DEP; DMP; abnormal sexual differentiation; hypospadias; risk assessment.

Exposure to synthetic environmental chemicals (e.g., DDT and its metabolites, especially p,p′-DDE, alkylphenol ethoxylates, PCBs, and dioxins) produces reproductive problems in a variety of vertebrate species via endocrine mechanisms (Ankley and Giesy, 1998; Colborn and Clement, 1992; Giesy and Snyder, 1998; Gray, 1991, 1992; Monosson, 1997; Van Der Kraak *et al.,* 1998). Naturally occurring environmental chemicals (e.g., phytoestrogens and estrogenic mycotoxins) induce infertility in domestic animal species (Adams, 1989; Ueno, 1985) and can alter human reproductive function (Cain, 1960). Concerns over these findings have been compounded by a series of publications suggesting that *in utero* exposure to environmental chemicals may have contributed to the reported decline in human sperm counts, the increased incidences of urogenital malformations (e.g., hypospadias, testicular cancer, and undescended testes) (Carlsen *et al.,* 1992), and altered sex ratio over the last 40–50 years. Sharpe and Skakkebaek (1993) hypothesized that *in utero* exposure to environmental estrogens could be responsible for the increased incidences of these alterations. This hypothesis is biologically plausible because hormones play critical roles as regulators of development in vertebrates, and exposure to hormonally active toxicants during sexual differentiation is known to produce abnormal reproductive phenotypes in humans and other animals (Gray, 1992; Schardein, 1993).

During mammalian sex differentiation, the androgens, testosterone (T), and the T metabolite dihydrotestosterone (DHT),

The research described in this article has been reviewed by the National Health and Environmental Effects Research Laboratory, U. S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

[1] To whom correspondence should be addressed. Fax: (919) 541-4041. E-mail: gray.earl@epa.gov.

produced by the fetal/neonatal male during sexual differentiation, are critical determinants of the male phenotype (Wilson, 1978). Differentiation of the Wolffian structures (e.g., the epididymis, vas deferens and seminal vesicles) is T mediated, while masculinization of the prostate and external genitalia is controlled by the more potent androgen DHT. In the central nervous system of the rat, some sex dimorphisms result from the action of estradiol, locally produced by the conversion of T by aromatase, while others, like play behavior, appear to be dependent upon androgens themselves (Gray, 1992). It appears that both T and DHT play important roles in the development of the brain in many mammals, including nonhuman primates, and other tissues like the spinal cord and levator ani muscles (T dependent). These developmental events can be altered by exposure to chemicals that bind AR and act either as hormone agonists or antagonists. Androgenic substances, like Danazol or methyltestosterone, masculinize human fetal females (i.e., female pseudohermaphroditism) (Schardein, 1993). Progestins act both as androgen antagonists in male offspring, demasculinizing them such that they display ambiguous genitalia with hypospadias (Schardein, 1993), and androgen agonists in females, virilizing the external genitalia. Laboratory studies of chemicals that inhibit or mimic the action of androgens also produce predictable alterations of sex differentiation in rodents (reviewed by Gray *et al.*, 1999a).

In the last few years, transgenerational studies on developmental reproductive toxicity of the phthalates have demonstrated that several of these produce malformations in male rat offspring after *in utero* and neonatal treatment. A NOAEL of 50 mg/kg/d has been identified for DBP (Mylchreest *et al.*, 1998, 1999, 2000), but other phthalates have been less thoroughly studied. It has been shown that the plasticizer DEHP also alters sexual differentiation in male rats in an antiandrogenic manner much like the effects of DBP (Gray *et al.*, 1999a).

Administration of DEHP to pregnant rats by gavage at 750 mg/kg/day during the period of sexual differentiation (gestational day [GD] 14 to postnatal day [PND 3]) markedly demasculinizes and feminizes the male offspring. Treated male offspring display femalelike anogenital distance (AGD) at birth, retained nipples, cleft phallus with hypospadias, undescended testes, blind vaginal pouch, epididymal agenesis, and small to absent sex accessory glands. Arcadi *et al.* (1998) observed testicular histological alterations and testis weight reductions when DEHP was administered at dosage levels in the drinking water at 32.5 μl/l or 325 μl/l (estimated as 3 and 30 mg/kg/day) to the dam during gestation and lactation. Poon *et al.* (1997) also observed testicular effects in the male rat at relatively low dosage levels (3.7 mg DEHP/kg/day was identified as a NOAEL and 37 mg/kg/day was the LOAEL) when they administered DEHP in the diet for 90 days to young male rats (weighing 105–130 g at the start of the study). DEHP treatment caused mild vacuolation in the Sertoli cells at 36.8 mg/kg/day (500 ppm in the diet considered the LOAEL) in 7/10 males and mild seminiferous tubular atrophy at 5000 ppm in 9/10 males. In addition, subtle effects were seen in the testis of males in both the NOAEL" dose group and the group below the NOAEL. Four of ten males in each low-dose group (5 ppm or 0.37 mg/kg/day and 50 ppm or 3.7 mg/kg/day, which was used as the NOAEL) displayed minimal Sertoli cell vacuolation. The incidence of minimal Sertoli cell vacuolation (8/20) is significantly greater than the incidence in the control group (0/10) $p < 0.03$, Fisher's Exact Test) and is consistent with the observation of increasingly severe Sertoli cell lesions at 500 and 5000 ppm.

Using the data from Poon *et al.* (1997), the Organization for Economic Cooperation and Development (OECD) estimated margin of exposure (MOE) of 19 for DEHP for children mouthing toys containing this chemical, whereas for DINP the value was 75 (EC 11/98 Opinion on Phthalate Migration, found at http://europa.eu.int/comm/food/fs/sc/sct/out19_en.html). A survey of phthalates revealed that 32 of 63 toys sampled contained DEHP, and 44 of 63 contained DINP. As a consequence of the low MOE, the European commission proposed a ban of several phthalates in childcare articles and toys (found at http://europa.eu.int/comm/dg03/press/1999/IP99829.htm), including DINP, DEHP, DNOP, DIDP, DBP, and BBP (Annex 1 to Directive 76/769/EEC and Annex IV of Directive 88/378/EEC; also, Environment News Service, 10/26/99, Environmental Data Services Ltd., London. E-mail: envdaily@ends.co.uk). The European Commission did not use the data of Arcadi *et al.* (1998) for the calculation of an MOE, and they did not include exposures to DEHP via other routes, although they did acknowledge that mouthing of toys might not be the major source of DEHP (see above EC references). The data from Arcadi *et al.* (1998) were considered as supportive of the effects reported by Poon *et al.* (1997).

Although *in utero* DEHP treatment alters androgen-dependent tissues, it does not appear to act as an androgen receptor (AR) antagonist like the pesticide vinclozolin (Gray *et al.*, 1994; Kelce *et al.*, 1994). *In vitro* studies found that neither DEHP nor the primary metabolite MEHP compete with androgens for binding to the AR at concentrations up to 10 μM (companion paper by Parks *et al.*, 2000). In contrast to their lack of ability to bind to the AR, DEHP inhibits fetal Leydig cell testicular testosterone synthesis, reducing fetal male testosterone concentration to a female level from GD 17 to PND 2 (Parks *et al.*, 2000).

In the current study, we compare the ability of six phthalate esters administered at one dose level to alter development of the male reproductive system, including the external genitalia (cleft phallus, hypospadias, vaginal pouch, and anogenital distance; DHT dependent), areola and nipple retention (DHT dependent), development of the ventral prostate (DHT dependent), seminal vesicles (T dependent), epididymides (T dependent), levator ani plus bulbocavernosus muscles (T dependent), and gubernacula (T dependent). We hypothesized that these

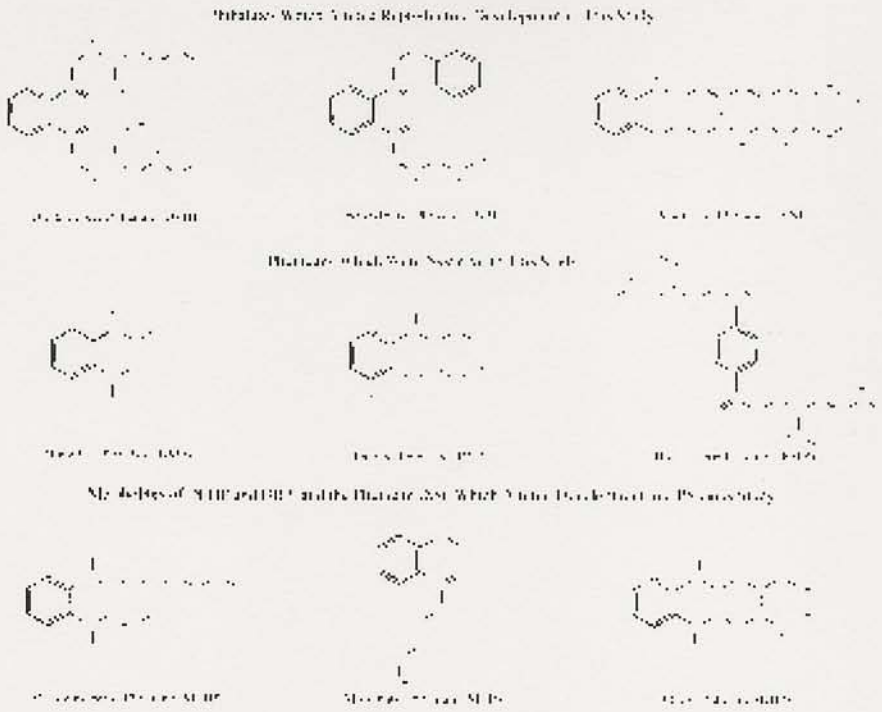


FIG. 1. Structure of phthalate esters which did (upper panel) or did not (middle panel) alter sexual differentiation of the male rat. Structure of dibutyl phthalate (DBP), which also alter sexual differentiation of the rat and rabbit, and the presumptive active metabolites of DBP (MBP) and DEHP (MEHP).

phthalates would display the same structure–activity relationship as described by Foster *et al.* (1980) for the effects of short-term treatment with phthalate esters on the testis of the peripubertal male rat. We expected that phthalate esters with ester side chains four to six carbons in length in the ortho configuration would be antiandrogenic *in utero* (DEHP and BBP), while DOTP (C6-para position; also known as diethylhexyl terephthalate-DEHT), DEP (C2-ortho position), and DMP (C1-ortho position) would be inactive. In addition, we anticipated that mixture DINP would be weakly positive as a consequence of the presence of low amounts of phthalate with the ester groups (C6-7 long in the ortho position) (Fig. 1).

MATERIALS AND METHODS

Animals. Ninety-day-old nulliparous female Sprague-Dawley rats were mated overnight (matings were confirmed by the presence of a sperm-positive vaginal smear) on proestrus and shipped from Charles River Breeding Laboratory, Raleigh, NC, on GD 2. Upon arrival, females were housed individually in clear polycarbonate cages (20 × 25 × 47 cm) with heat-treated (to eliminate resins), laboratory-grade pine shavings as bedding (Northeastern Products, Warrensburg, NY). Animals were maintained on Purina Rat Chow (5001) and filtered tap (Durham County) water (checked monthly for bacteria and every 4 months for pesticides and heavy metals) *ad libitum* in a room with a 14:10-h photoperiod (L/D, lights off at 11:00 AM, EST) and temperature of 20–24°C with a relative humidity of 40–50%.

Maternal Dosing. Laboratory-grade corn oil (CAS # 8001-30-7, Sigma lot # 107h1649) was the vehicle chosen to prepare all dosing solutions. In the first block, DEHP (CAS # 117-81-7, Sigma lot # 106h3487, 99% purity), BBP (CAS # 85-68-7, Aldrich lot # 08523jq, 98% purity), DEP (CAS # 84-66-2, Sigma lot # 48h3537, 99% purity), DINP (CAS # 68515-48-0, Aldrich lot # 03005TR, purity = technical), and DMP (CAS # 131-11-3, Aldrich lot # 08812jy, 99% purity) were administered by gavage at 0 or 750 mg/kg/day in 2.5 μl of corn oil/g body weight from GD 14 (sperm detected on GD 1) until PND 3 (postcoital day 23 = PND 1). Dams (for *n* see Table 1) were assigned to treatments in a manner that provided similar means and variances in body weight before dosing was initiated. The dose administered included daily adjustments based on individual maternal weight changes throughout the dosing period. A second block was conducted to repeat the positive effects seen with DINP and BBP and to examine, for the first time, the effects of DOTP. In this block, pregnant rats per group were dosed, as above, with the vehicle, DEHP, BBP, DINP, or DOTP (CAS # 6422-86-2, Aldrich lot # 09704tr, 98% purity) in the vehicle.

Neonatal and Infantile Data. Body weight and AGD were measured in offspring on PND 2 (as per Gray *et al.*, 1999a,b) and one randomly selected male was necropsied from each litter in order to measure paired testes weight and histology (semithin plastic sections; for methods see Parks *et al.*, 2000). In the second block, a second randomly selected male was removed for histological examination of the testes (methods as per Parks *et al.*, 2000) in the control, DEHP, and BBP groups (Parks *et al*, 2000) on PND 3.

At 9–10 days of age, the inguinal region of each male pup was examined for suspected hemorrhagic testes; at 13 days of age they were examined for the presence of areolas/nipples, described as dark focal areas lacking hair (called an areola) with and without a nipple bud (in a blinded manner). Testes from control and DEHP-treated male pups were placed in Bouin's fixative for 24 h, after which they were rinsed and stored in 70% alcohol, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined by Dr. N. R. Veeramachaneni for histopathological lesions.

At PND 28 male pups were weaned and housed in groups of 2 or 3 with littermates. After weaning, all male pups were examined daily for the onset of puberty, as indicated by preputial separation, and the data were analyzed using litter means.

Mounting behavior and intromissions. Prior to necropsy, a subset of adult male rats from the control ($n = 3$) and DEHP ($n = 6$) groups were examined

TABLE 1
Maternal and Neonatal Pup Effects of Various Phthalate Esters (PE) after Perinatal Maternal Exposure (GD 14–PND 3) to 0.75 g PE/kg/day on the Sprague-Dawley Rat

	Control	BBP	DEHP	DINP	DEP	DMP	DOTP
Numbers of dams assigned to this study Block 1/ Block 2	9/10	5/8	7/9	6/8	5/0	5/0	0/8
Numbers of dams died	0	0	0	0	2	1	0
Numbers of dams with live pups at day 2	19	13	16	14	3	4	8
Numbers of dams with live pups at weaning	19	11	16	14	3	4	8
Maternal weight GD 14 Block 1 (g)	303 ± 5.2	302 ± 4.8	303 ± 7.1	305 ± 5.2	301 ± 4.3	302 ± 4.7	—
Maternal weight GD 14 Block 2 (g)	310 ± 4.0	309 ± 5.1	310 ± 3.9	309 ± 4.8	—	—	309 ± 4.8
Maternal weight gain to GD 21 (g)	104 ± 3.7	110 ± 5.7	79.3** ± 4.4	89.6* ± 5.0	106 ± 7.1	102 ± 5.6	104 ± 5.6
Maternal weight gain to PND 3 (g)	15.4 ± 3.6	7.5 ± 3.3	0.12** ± 3.0	10.5 ± 4.8	23.7 ± 4.9	22.5 ± 5.8	3.2 ± 4.8
Number of Live pups Day 1	13.5 ± 0.4	12.8 ± 1.1	13.3 ± 0.4	12.4 ± 0.7	14.7 ± 0.3	13.5 ± 0.9	13.1 ± 0.6
Litter mean pup weight at birth (g)	6.84 ± 0.06	5.78** ± 0.18	5.78** ± 0.22	6.93 ± 0.11	6.60 ± 0.13	6.59 ± 0.24	6.80 ± 0.14
Litter mean male pup weight at weaning (g) (no. pups/litters)	83.2 ± 1.4 (82/19)	87.8 ± 3.1 (48/11)	82.7 ± 2.9 (58/16)	85.7 ± 2.8 (52/14)	84.6 ± 8.5 (14/3)	80.7 ± 2.1 (21/4)	82.3 ± 4.5 (40/8)
Litter mean male age at puberty (PPS)	43.5 ± 0.2 (81/19)	44.8 ± 1.0 (34/10)	44.5 ± 0.9 (37/10)	42.9 ± 0.5 (49/14)	42.7 ± 1.1 (13/3)	44.4 ± 0.8 (21/4)	42.8 ± 0.4 (40/8)
Incomplete PPS due to genital malformations (No. without PPS/ no. males observed)	0	9/46**	19/56**	0	0	0	0

Note. Values are litter means ± standard errors.
* Indicates value differs from control by $p < 0.05$.
** Indicates $p < 0.01$.

for the display of male mating behavior. The males examined from the DEHP group included those with ($n = 2$) and without ($n = 4$) cleft phallus and hypospadias. Each male was paired with a sexually receptive female rat for 30 min during the dark phase of the animal's diurnal cycle or until he displayed approximately 10 mounts (with or without intromission). A larger number of animals was not observed because DEHP animals displayed normal mounting behavior.

Necropsy. At 3–5 (Block 1) or 4–7 (Block 2) months of age, males were killed by decapitation within 15 s of removal from the home cage in a separate room to control for the stress on hormone levels (see Table 2 for the numbers of animals observed). The order of necropsy was generally balanced with respect to treatments. Blood was collected for determination of serum testosterone (T) levels and the males were necropsied. The ventral surface of each male was shaved and examined for abnormalities, including the number and location of retained nipples, cleft phallus, vaginal pouch, and hypospadias. The animals were examined internally for undescended testes (with and without cranial suspensory ligaments), atrophic testes, epididymal agenesis, prostatic and vesicular agenesis, and abnormalities of the gubernacular cord. Weights taken at necropsy included body, pituitary, adrenal, kidney, liver, ventral prostate, levator ani plus bulbocavernosus muscles, seminal vesicles (with the coagulating gland and their fluids), testes, and epididymides. One testis was used for testis spermatid head count (TSHC) and one cauda epididymis was used to determine total and cauda epididymal sperm reserves as previously described (Gray *et al.*, 1999b) for some of the rats.

Reproductive organs weights were taken from almost every male from each litter (BBP = 45 pups/11 litters, CON = 77 pups/19 litters, DEHP = 41 pups/15 litters, DEP = 12 pups/3 litters, DINP = 52 pups/14 litters, DMP = 21 pups/4 litters, DOTP = 39 pups/8 litters); nonreproductive organs were not weighed in all males due to a lack of effects on these organs in the first block (BBP = 30 pups/10 litters, CON = 45 pups/17 litters, DEHP = 23 pups/13 litters, DEP = 12 pups/3 litters, DINP = 29 pups/13 litters, DMP = 21 pups/4 litters, DOTP = 6 pups/6 litters). Some control ($n = 3$) and DEHP ($n = 4$) treated males were examined only for malformations, as above, but organ weights were not taken because these males were perfuse-fixed for histological examination of the testes, and one of the DEHP animals in this group had no siblings.

Statistical analysis of the data. Data were analyzed using a one-way ANOVA (treatments) model on PROC GLM from SAS available on the U.S. EPA IBM mainframe computer. *Post hoc* tests were conducted when the overall ANOVA was significant at the $p < 0.05$ level using the LSMEANS available on SAS. (The LSMEANS is a two-tailed *t*-test which is appropriate for *a priori* hypotheses derived from our previous work with DEHP and DBP). For statistical purposes, the numbers/group are the number of litters, not the number of pups. Because the data analysis for maternal, pup, and adult necropsy data as two blocks or as a single study with two blocks yielded identical results, the results are presented with the blocks pooled. AGD, growth, pubertal, and necropsy data were analyzed at each age by ANOVA using litter mean values rather than individual values. AGD and organ weight

TABLE 2
Effects of Various Phthalate Esters on Male SD rat Offspring Body and Organ Weights and Serum Testosterone Levels after Perinatal Maternal Exposure (GD 14–PND 3) to 0.75 g/kg/day

	Control	BBP	DEHP	DINP	DEP	DMP	DOTP
No. litters examined for repro/nonrepro organ wts.	19/17	11/10	15/15	14/13	3/3	4/4	8/6
No. litters examined for malformations	19	11	16	14	3	4/4	8
No. males: organ wts. (repro/nonrepro)	77/45	45/30	41/23	52/29	12/12	21/21	39/6
No. Males examined for malformations	80	45	45	52	12	21	39
Testes (mg)	3508 ± 53	2718* ± 300	2689* ± 241	3511 ± 72	3403 ± 128	3523 ± 86	3584 ± 91
LABC (mg)	1275 ± 22	843* ± 74	851* ± 69	1211 ± 27	1287 ± 34	1234 ± 39	1301 ± 42
Seminal vesicle plus CG (mg)	1857 ± 45	1154* ± 136	1184* ± 215	1725 ± 46	1637 ± 99	1798 ± 101	2082 ± 89
Ventral prostate (mg)	685 ± 21	398* ± 54	511* ± 59	678 ± 25	642 ± 9.0	677 ± 15	727 ± 31
Glans penis (mg)	108 ± 1.3	88* ± 4.0	81* ± 4.9	106 ± 1.8	111 ± 4.8	105 ± 5.3	110 ± 1.8
No. nipples per male	0	5.1* ± 0.9	6.3* ± 1.1	0.11 ± 0.09	0	0	0
Paired epididymides (mg)	1293 ± 18	966* ± 67	945* ± 82	1269 ± 21	1229 ± 25	1251 ± 26	1343 ± 23
Cauda epididymis (mg)	312 ± 5.3	182* ± 21	180* ± 29	307 ± 6.8	288 ± 11	295 ± 9.8	330 ± 7.7
Caput-corpus epididymis (mg)	333 ± 5.3	245* ± 20	278* ± 18	321 ± 7.5	315 ± 4.8	318 ± 12	343 ± 6.5
Body weight (g)	613 ± 17	607 ± 30	606 ± 16	609 ± 19	547 ± 13	547 ± 15	659 ± 18
Paired kidney (mg)	3754 ± 83	3510 ± 146	3516 ± 124	3744 ± 100	3662 ± 233	3635 ± 116	3959 ± 112
Liver (g)	20.1 ± 0.8	18.8 ± 0.9	18.5 ± 0.9	21.0 ± 0.9	18.9 ± 1.3	19.9 ± 0.8	22.3 ± 1.5
Pituitary (mg)	14.9 ± 0.45	15.0 ± 0.53	15.0 ± 0.54	15.2 ± 0.51	14.2 ± 1.0	14.6 ± 0.39	14.0 ± 0.34
Adrenals (mg)	58.0 ± 1.9	57.3 ± 1.9	56.7 ± 1.9	53.7 ± 1.8	50.2 ± 2.9	54.2 ± 3.4	59.2 ± 2.1
Serum T (ng/ml)	1.15 ± .13	1.45 ± 0.24	1.33 ± 0.16	1.89 ± 0.51	1.79 ± 0.37	1.40 ± 0.20	0.79 ± 0.12

Note. Weight data are litter mean values ± standard errors.
* Indicates value differ from control by $p < 0.01$.

data were analyzed with body weight as a covariate. Analysis of the epididymal, seminal vesicle, and ventral prostate weight data did not include zero values when complete agenesis was displayed. In addition, sex accessory tissue weight data also were excluded if the glands appeared infected (being impacted and/or discolored). The percentage of infant males displaying areolas (on a litter mean and individual basis) and the numbers of nipples/areolas per male were analyzed for treatment effects. Retained nipple data collected in adult males at necropsy were similarly analyzed. Categorical data (numbers of pups affected versus total pups and numbers of litters affected out of the total numbers of litters) were analyzed using Fisher's Exact Test or Chi Square Test, as appropriate.

RESULTS

Maternal and Litter Effects

None of the phthalate ester treatments caused maternal death or overt maternal toxicity. Two DEP- (*n* reduced from 5 to 3 litters) and one DMP-treated (*n* reduced from 5 to 4 litters) dams died from dosing errors. DEHP and DINP treatments modestly reduced maternal weight gain, but maternal body weights were not significantly reduced at any time (Table 1). By the end of dosing on PND 3, only DEHP-treated dams displayed a significant reduction in weight change from the start of dosing. All the dams were pregnant and delivered live pups. The litter from one BBP-treated dam did not survive to 2 days of age, and another did not have any male pups at weaning (*n* reduced from 13 to 11 litters). The numbers of live pups at birth were not reduced by any of the phthalate ester treatments (Table 1), whereas pup weights were significantly reduced in the DEHP and BBP groups (Table 1).

Alterations in Neonatal and Infant Male Offspring

AGD, with or without covariance adjustment for body weight, was significantly reduced in male, but not female, pups in the BBP and DEHP treatment groups by about 30% at 2 days of age (Fig. 2). In contrast, both sexes displayed similar reductions in body size at this age (about 15%). These two treatments also produced a significant reduction in paired testes weights (about 35%) examined in one male per litter at this time (Fig. 3). At 8–9 days of age, several males (seven from three litters) in the DEHP group displayed hemorrhagic testes, as indicated by a darkening of the inguinal region. This also was observed in one male from the DINP treatment group. (This animal was not necropsied at this time. Hence, the observation should be considered as suspected rather than confirmed hemorrhagic testis, as several male pups in this litter died during lactation and were not necropsied).

Histological examination of some of the testes from DEHP-treated animals revealed focal interstitial hemorrhage (Fig.

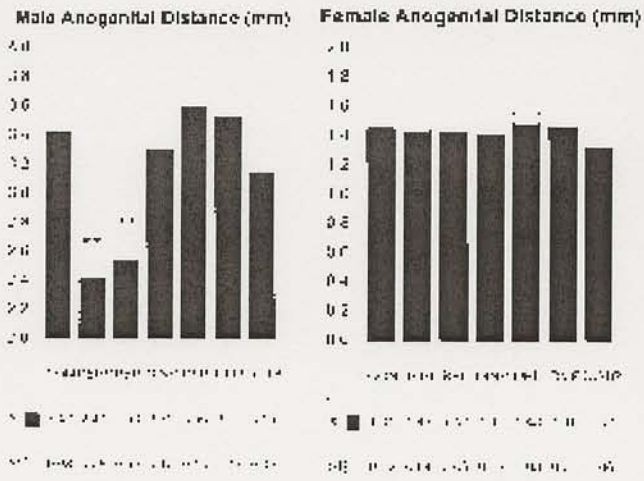


FIG. 2. Maternal DEHP and BBP treatments (0.75 g/kg/day from day 14 of gestation to day 3 of lactation) reduced anogenital distance (mm) in male but not female rat pups. Both of these phthalate esters reduced the sexual dimorphism in this trait by about 50%; $^{**}p < 0.001$ for litter-based values, including body weight as a covariate in the analysis of these data.

4A), phagocytosis of extravasated erythrocytes, and aggregation of cells in the interstitium (Fig. 4B) by PND 2 and 3. Examination of thin sections of cell aggregates in the interstitium confirmed this phagocytic activity by the presence of erythrophagosomes. Occasional band cells and atypical cells with meandering nuclei were also evident. By PND 9–10, although some testes had focal granulomata (indicating that the hemorrhage was limited to focal areas and contained) (Fig. 4C), others manifested extensive coagulative necrosis, a sequel of hemorrhage (Fig. 4D). The latter were the grossly visible hemorrhagic testes. A closer examination of the luminal contents of blood vessels elsewhere in animals with hemorrhagic testes revealed presence of reticulocytes, indicating severe loss of erythrocytes, and therefore premature release of reticulocytes into circulation. The infarcted areas of testis (resulting from hemorrhage and coagulative necrosis) were characterized by loss of seminiferous epithelial architecture and deposits of hemosiderin. The natural progression of this lesion would be fibrosis, resulting in complete atrophy or even complete disappearance of testicular tissue. Notably, differentiating germ stem cells were also affected by DEHP. In PND 2 and PND 3 DEHP-treated animals, multinucleated gonocytes containing as many as 3–5 nuclei and some undergoing degenerative changes were observed (Figs. 4A and 4B).

Perinatal BBP, DEHP, and DINP treatments significantly induced areolas in male offspring (Fig. 5), based on analysis of either litter means or individual values. Body weights in BBP and DEHP animals were not reduced as compared to controls, either at weaning (28 days of age) or later in life (Tables 1 and 2).

Pubertal Landmarks

The age at preputial separation (PPS) was not delayed by phthalate ester treatments except in extremely malformed males in the BBP and DEHP groups (Table 1). In incomplete PPS, males typically displayed a permanent thread of tissue (persistent frenulum), a condition resulting from failure of normal liquefaction of the ectodermal ingrowth between the glans penis and prepuce. It is apparent that such alterations in PPS are not related to puberty per se but rather reflect abnormal differentiation of the prepuce. For this reason, data from the malformed males were not included in the analysis of the age at puberty.

Male Rat Sexual Behavior

When DEHP and control male rats were paired with sexually receptive females to examine male mating behavior, 4/6 treated males displayed mounts with pelvic thrusts versus 2/3 controls. Although treated males with malformations of the penis were unable to attain intromission, treated males with normal external genitalia displayed normal intromissions. Hence, these data do not support the hypothesis that PEs alter sexual differentiation of CNS with respect to male rat sexual behavior. However, our data on the levator ani/bulbocavernosus muscles would suggest that differentiation of other components of the nervous system such as the spinal cord are likely abnormal in BBP- and DEHP-treated animals.

Necropsy Data

Malformations. The numbers of animals and litters examined for malformations are given in Table 1. DEHP, BBP, and

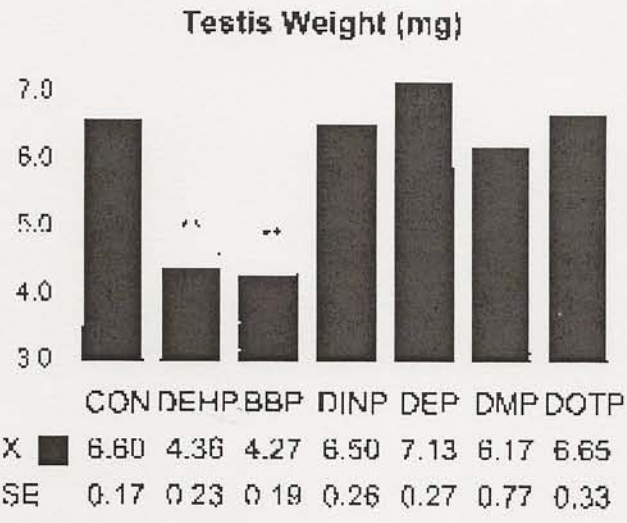
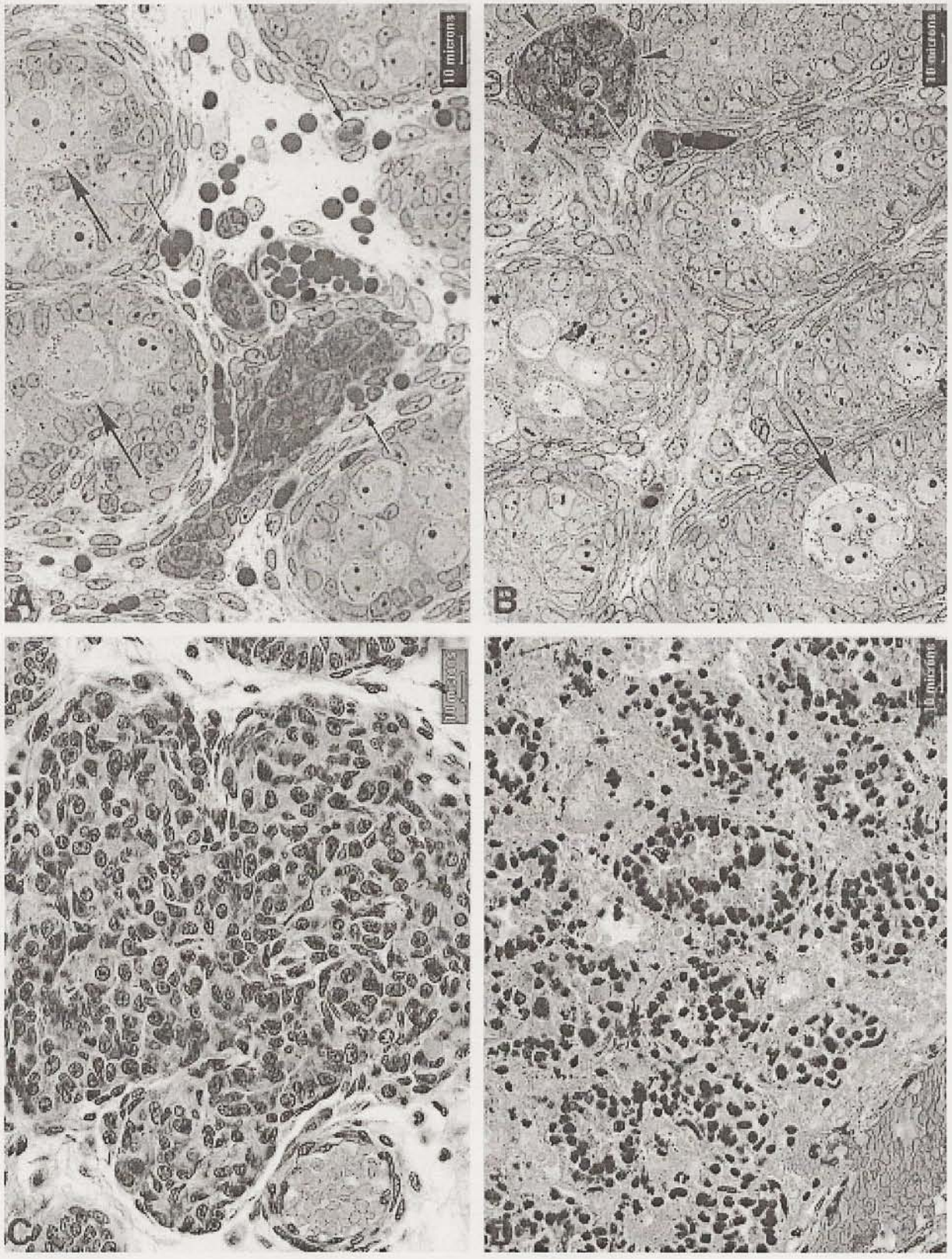


FIG. 3. Maternal DEHP and BBP treatments (0.75 g/kg/day from day 14 of gestation to day 3 of lactation) reduced paired testes weights in male offspring (1/litter) at 2 days of age, including body weight as a covariate in the analysis of these data.


10 microns
A
10 microns
B
C
10 microns

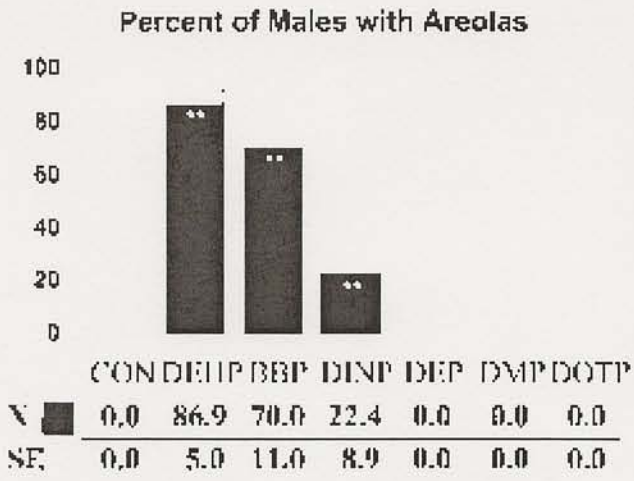


FIG. 5. Maternal treatment with DEHP, BBP, or DINP (0.75 g/kg/day from GD 14 to day 3 of lactation) significantly increased the incidence of male offspring with areolas (with and without nipple buds); $^{**}p < 0.01$ based on litter means analysis.

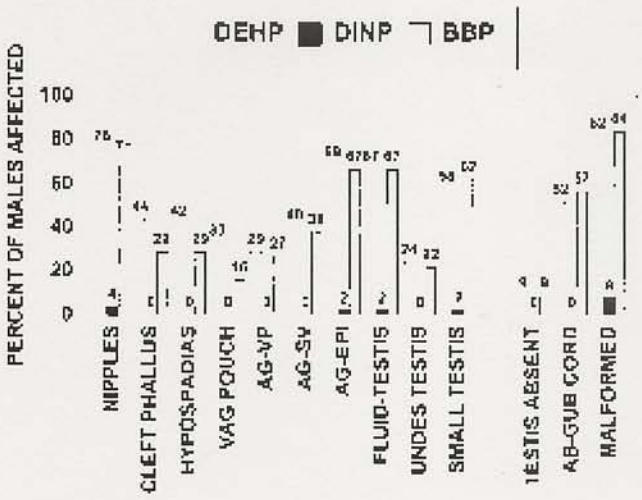


FIG. 6. Maternal treatment with DEHP, BBP or DINP (0.75 g/kg/day from GD 14 to day 3 of lactation) significantly increased the incidence of male offspring with malformations of the androgen-dependent organs and testes on an individual (as shown here) or litter basis.

DINP treatments induced malformations in male offspring (Fig. 6). DEHP and BBP were of equivalent potency (82 vs 84%, respectively; $p < 0.0001$ vs control). Both were considerably more potent than was DINP, which induced a significant level (7.7%) of reproductive malformations on an individual animal basis (4/52 pups, $p < 0.05$ by Chi Square on an individual basis and $p < 0.06$ on a litter basis for 3/14) versus control (0/19 litters and 0/80 individuals).

The variability from animal to animal in the types of malformations displayed and the severity of these lesions was quite remarkable. Some litters were clearly more or less affected (or unaffected) than others and it was not unusual to find litters in which some siblings were mildly affected, displaying a single malformation, while others were severely affected with 12 malformations. Within the affected treatment groups, affected animals often displayed very different constellations of malformations. Every reproductive tissue that we examined was impacted to some degree by DEHP and BBP treatments. Even the 4 affected (out of 52) DINP males displayed diverse malformations.

The numbers of permanent nipples ranged from 1–14 (12 being the number typically found in a female rat vs zero in a male). Most DEHP and BBP and 2/52 DINP males displayed permanent nipples. In BBP- and DEHP-treated males, clefting of the phallus was complete in many males, resulting in hypospadias, while on occasion the phallus was partially cleft with epispadias. Cleft phallus and hypospadias were often concurrent with a vaginal pouch. Some DEHP- and BBP-treated males displayed complete agenesis of the ventral prostate and unilateral (one horn) or complete agenesis of the seminal vesicles and coagulating glands. Although not systematically recorded, it was noted that DEHP and BBP males often displayed agenesis of the bulbourethral glands.

Several testicular malformations were seen. These likely resulted from several different mechanisms involving:

- hemorrhage, granuloma, fibrosis/complete or partial replacement of testicular tissue by connective tissue
- pressure atrophy associated with epididymal agenesis
- nondescent of the testis associated with abnormalities of the gubernacula or presence of cranial suspensory ligaments.

Small (classified as less than 1.2 grams, which was smaller than any control testis) and atrophic [classified as less than 750

FIG. 4. Photomicrographs of testicular sections of rats exposed to DEHP at 750 mg/kg/day between GD 14 and PND 3 showing the progression of the testicular lesions from PND 2 to PND 10. (A) Testis at PND 2 stained with toluidine blue. Hemorrhage is seen, as evidenced by extravasated erythrocytes in the interstitium. Note aggregation of cells predominantly appearing to be fibroblasts and histiocytes. Phagocytosis of erythrocytes and erythrophagosomes are seen (arrows). Also note the presence of multinucleated gonocytes in seminiferous tubules (large arrow). (B) Testis at PND 3 stained with toluidine blue. Note encapsulated clustering of cells in the interstitium (arrow heads). Phagocytosis (arrow) and hemosiderin resulting from degraded erythrocytes within the cells are evident. Also note multinucleated gonocyte in the seminiferous tubule (large arrow). (C and D) Testes at PND 10 stained with hematoxylin and eosin. Formation of focal granulomata in the interstitium (C) and coagulative necrosis of the entire testis (D). When hemorrhage was limited to focal areas of the interstitium, localized granulomatous reaction was observed with remaining areas of the testis appearing normal; if the hemorrhage was multifocal and extensive, coagulative necrosis involving both interstitial and seminiferous epithelial elements was observed.

mg (half control size)] testes were seen in DEHP, BBP, and DINP male offspring at necropsy (Fig. 7, DINP small and atrophic testes). Several males (about 9%) in the DEHP and BBP groups displayed complete unilateral testicular agenesis/atrophy. In one case (DEHP group), both testes were absent. This lesion was not present in fetal or 2- to 3-day-old males (Parks *et al.*, 2000).

Flaccid, fluid-filled testes were seen in the DINP, DEHP, and BBP groups. In some cases the fluid-filled testes were atrophic, lacking sperm, whereas in other cases testis size was normal or enlarged but still devoid of sperm. Fluid-filled testes were typically associated with ipsilateral agenesis of the epididymis.

Undescended testes were displayed by DEHP- and BBP-treated males. Some of these testes were free floating, completely lacking a gubernaculum, while others were attached to a thin elongated gubernaculum (ranging from 10 to 50 mm in length vs a normal length of less than 5 mm). Some scrotal testes lacked any gubernacular tissue, indicating that abdominal pressure and muscle tone around the inguinal ring were sufficient to induce scrotal retention of the testes, which then supported spermatogenesis. For example, one male displayed retained nipples, a small ventral prostate, and bilateral agenesis of the gubernacular cords, while the testes and all other tissues were normal. In one case of bilateral agenesis of the gubernaculum, the left testis had descended into the right scrotal sac, while the right testis was in the abdominal cavity. In another animal, both testes had descended into the contralateral scrotal sacs.

Specific comments on DINP males. As indicated above, DINP induced a significant level (7.7%) of reproductive malformations on an individual animal basis (4/52, $p < 0.05$ by Chi Square) versus control (0/19 litters and 0/80 individuals). In addition, another male from one of the above litters in the DINP group displayed bilateral testicular atrophy, but these testes were not fluid filled or flaccid (Fig. 7). The fourth affected male in the DINP group was from a third litter and displayed unilateral epididymal agenesis with hypospermatogenesis and scrotal fluid-filled testis devoid of spermatids (Fig. 8). Another male was suspected of displaying a hemorrhagic testis at 9 days of age, but as 4 of 7 males died in this litter before 13 days of age, this effect was not confirmed by necropsy. In the DINP group, 2/52 males (from 2/14 litters) displayed nipples (no. of nipples = 1 and 6 for each of the two males) (Fig. 9). These are considered to be malformations, as permanently retained nipples represent a gross morphological alteration not normally seen in males of this species.

Body and organ weights. Body weight and non–reproductive organ weights were not affected by perinatal PE treatment (Table 2). In the BBP and DEHP-treated male offspring, every reproductive organ was significantly affected (testis weight and sperm production [data not shown], cauda and caput epididymal weights and caudal sperm numbers [data not shown], and seminal vesicle [plus coagulating gland with fluids], ventral prostate, glans penis, and levator ani plus bulbocavernosus muscle weights). As previously reported for DEHP (Gray *et al.*, 1999a), serum testosterone levels in adult male offspring at necropsy were not reduced by perinatal PE administration (Table 2), in marked contrast to the reductions in testosterone levels during fetal life.

DISCUSSION

The current study demonstrates that 0.75 g BBP and DEHP/kg/day severely alter sexual differentiation in the male rat with about equivalent potency. Although the effects of low dosages of BBP on male sexual differentiation are controversial (Ashby *et al.*, 1997; Sharpe *et al.*, 1995), it is clear that at 0.75 mg/kg BBP profoundly disrupts fetal sexual differentiation. DINP was about 10- to 20-fold less potent than BBP or DEHP, but it did display antiandrogenic activity. Better estimates of relative potency and determination of NOAELs will require execution of transgenerational dose-response studies. Although not tested here, several other studies have shown that DBP causes similar effects when administered during sexual differentiation (Gray *et al.*, 1999a; Mylchreest *et al.*, 1998; 1999; Wine *et al.*, 1997). The profile of malformations for DBP is nearly identical to that for DEHP and BBP.

Mechanisms of Antiandrogen Action

The severity of the effects on the T-dependent tissues resulting from exposure to DEHP and BBP (which include a high incidence of epididymal agenesis) differs from the profile of malformations seen with AR antagonists like flutamide (Imperato-McGinley, 1992), vinclozolin (Gray *et al.*, 1994, 1999b), and procymidone (Ostby *et al.*, 1999). When administered at a dosage level that produces an equivalent degree of hypospadias, the AR antagonists have much less of an effect on T-dependent tissues and the testis than do the PEs. It was the differences in the constellation of effects obtained with the PEs that led us (Gray *et al.*, 1999a) and Mylchreest *et al.* (1999) to hypothesize that although the PEs produce antiandrogenic effects during fetal life, they were not likely to be AR antagonists. Indeed, the recent work of Parks *et al.* (2000) on DEHP and of Foster *et al.* (1999) on DBP support this hypothesis. Our studies with DEHP (Parks *et al.*, 2000) demonstrate that this PE acts by inhibiting fetal rat T production, which in turn lowers testicular and whole-body T to female levels. Similar studies with DBP have shown that it also reduces fetal rat T levels during sexual differentiation (Foster *et al.*, 1999). Furthermore, the PEs and their monoester metabolites (DBP and MBP, DEHP, and MEHP; Parks *et al.*, 2000; Foster *et al.*, 1999) do not appear act as AR ligands *in vitro* at concentrations up to 10 μM.

Histological evaluation of the fetal and neonatal testis from DEHP- (Parks *et al.*, 2000) and DBP-treated (Foster *et al.*,

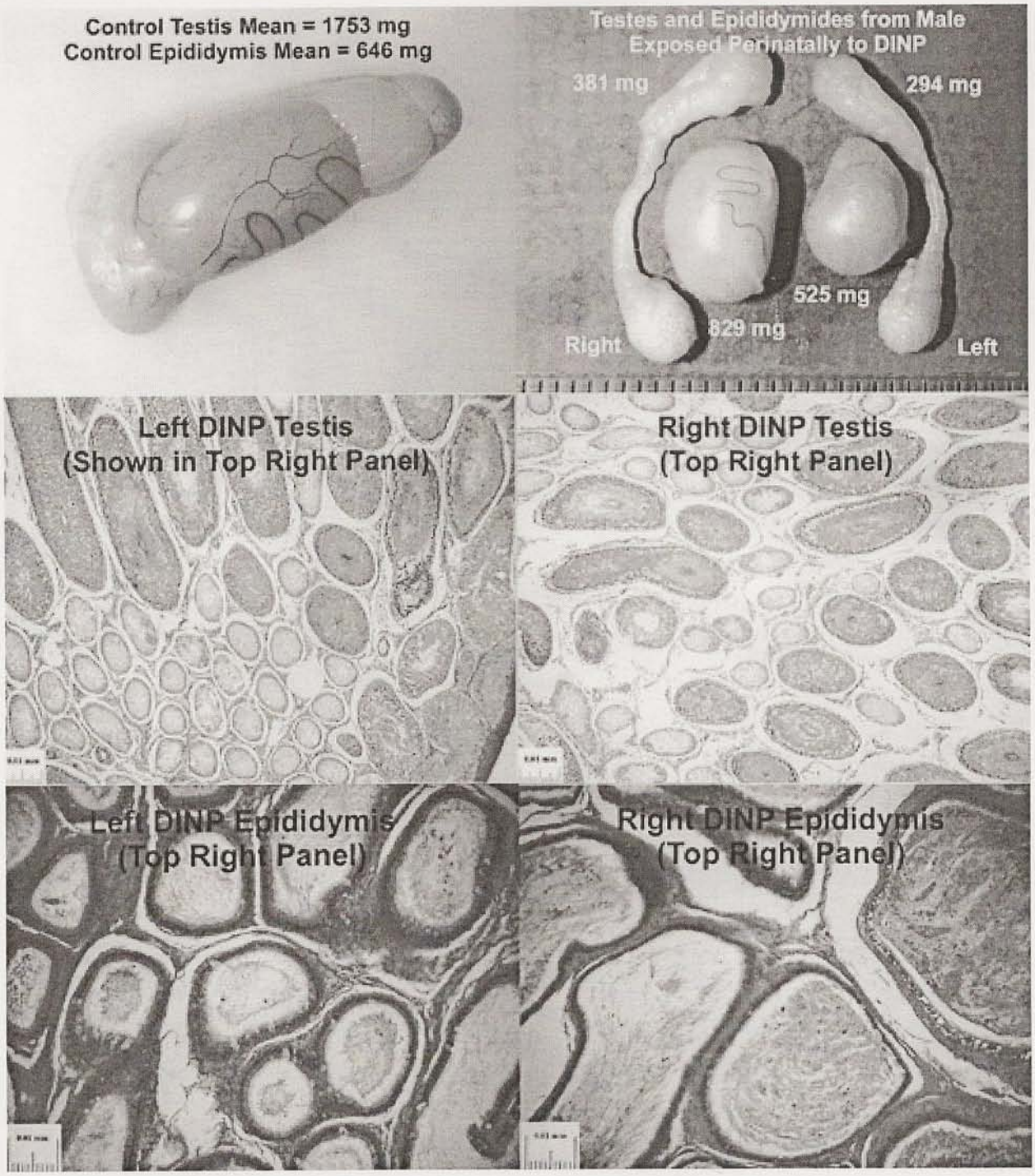


FIG. 7. Maternal treatment with DINP (0.75 g/kg/day from GD 14 to day 3 of lactation) significantly increased the incidence of male offspring with reproductive malformations on an individual (4/52, as shown here). The lesions seen in one male from the DINP group are displayed here. This male displayed paired testicular and epididymal atrophy and the anterior portion of the right testis appeared malformed (top right panel). A control testis and epididymis is provided in the top left for comparison. Both left and right testes from this DINP-treated male display atrophic tubules that lack any evidence of spermatogenesis (middle right and left panels, respectively). In addition, the lumen of both the left and right caudae epididymidis from this male contain cellular debris and reduced numbers of sperm.

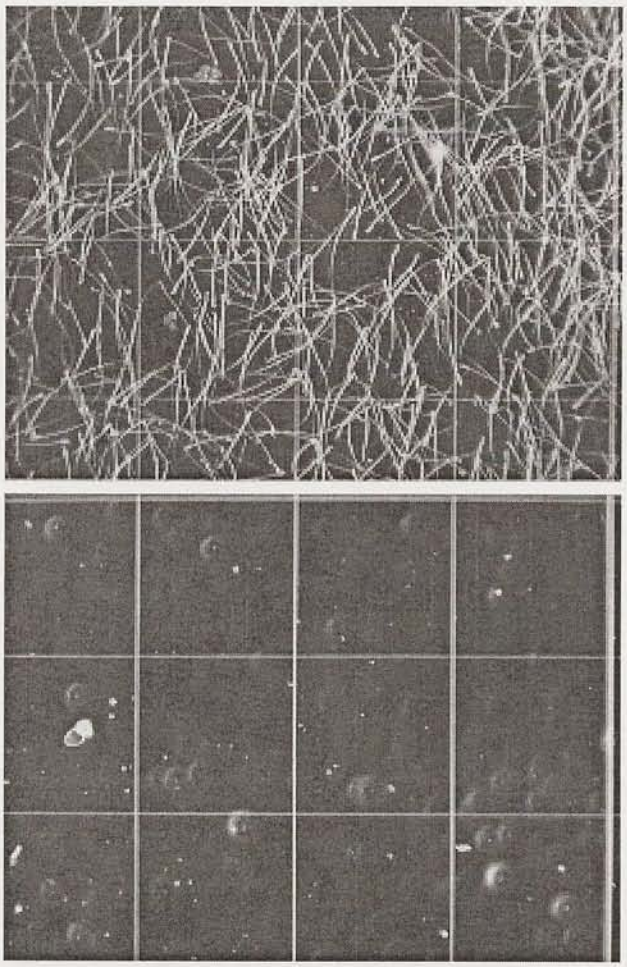


FIG. 8. Maternal treatment with DINP (0.75 g/kg/day from day 14 of gestation to day 3 of lactation) significantly increased the incidence of male offspring with reproductive malformations on an individual (4/52, as shown here). A photomicrograph of an undiluted sample of fluid from cauda epididymidis of a control (upper panel with sperm) and DINP male (lower panel), resulting from caput/corpus epididymal agenesis and a fluid-filled testis, is shown here.

1999) rats also supports the hypothesis that the fetal testis is directly affected by PEs during sexual differentiation. Examination of the testes of fetal and neonatal rats treated with DEHP revealed abnormal fetal Leydig cell (LC) morphology. When the testes were examined for 3β HSD staining by immunohistochemistry at GD 20 and at 2–3 days of age, fetal LCs of PE-treated males appeared to be increased in number in the interstitium of the testis. In addition, Mylchreest *et al.* (1999) observed LC adenomas in young adult male offspring after maternal treatment with DBP during pregnancy. Taken together, these studies indicate that the several PEs act in an antiandrogenic manner distinct from that previously seen with AR antagonists such as vinclozolin and procymidone. The fact that neither the age at puberty, except in malformed males, nor the level of serum T in adulthood are altered in most PE-treated males during adult life indicates that T production by adult LCs during peripubertal and adult life is not permanently affected by perinatal PE treatment, in contrast to the marked reductions in T levels and production seen in fetal LCs during gestation. Hence, the fact that the androgen-dependent tissues are reduced in size by perinatal BBP and DBP treatments is an indication of abnormal organization of these tissues; they have been permanently imprinted and demasculinized and do not have potential to respond normally to the activational effects of androgens after puberty.

Although most of the effects of PEs on sexual differentiation in the male rat can be attributed to antiandrogenicity, it is unclear if hemorrhagic testes would result from this mechanism of action. At 8 to 9 days of age, several PE-treated males displayed hemorrhagic testis. This effect was never seen in fetal or neonatal males but this condition is likely causally related and a precursor to the absence of testicular tissue that was displayed during adulthood by about 9% of the DEHP- and BBP-exposed male offspring. One treated male displayed complete bilateral agenesis/atrophy of the testes as an adult. The

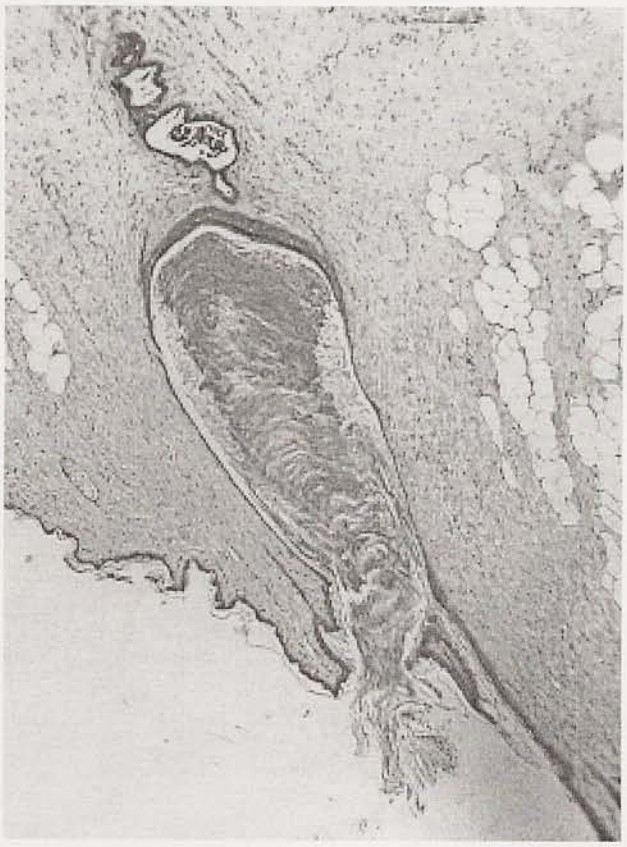


FIG. 9. Maternal treatment with DINP (0.75 g/kg/day from GD 14 to day 3 of lactation) significantly increased the incidence of male offspring with reproductive malformations on an individual (4/52, as shown here). One of the six permanent nipples seen in one male from the DINP group is shown here.

fact that this male did not display hypospadias, cleft phallus, vaginal pouch, or a female tract demonstrates that LC and Sertoli cell functions were normal for at least a portion of sexual differentiation. The fact that none of the PE-exposed male offspring displayed any Mullerian duct derivatives indicates that the Sertoli cells secreted sufficient antimullerian hormone to induce regression of the progenitors of the female reproductive tract. While it is clear that the PEs target the Sertoli cell in the pubertal and neonatal male rat, causing vacuolation and premature germ cell release, the Sertoli cells of DEHP-treated fetal male rats did not display such vacuolation. It remains to be determined if their function is altered. Alteration of Sertoli cell paracrine activity could result in the alterations of Leydig and germ cells caused by perinatal PE treatment.

Detection of Developmental Reproductive Toxicity of PEs

The data presented here for the PEs BBP, DEHP, and DINP and published data on DBP (Foster *et al.*, 1999; Gray *et al.*, 1999a; Mylchreest *et al.*, 1998; 1999) demonstrate that these PEs are developmental reproductive toxicants. This conclusion is in marked contrast to the conclusions of Koop and Juberg, 1999, who reported that DEHP and DINP were not specific developmental or reproductive toxicants. The discrepancy in interpretation arises from the fact that their review focused on a preponderance of negative standard teratology/developmental toxicity studies (all of which failed to identify PE-induced reproductive malformations), but failed to include some of the more recent transgenerational studies, which are positive. Several very competent scientists, using standard teratologic techniques, have reported the PEs are not teratogenic. DBP (Ema *et al.*, 1993; 1994), BBP (Ema *et al.*, 1992), DEHP (Hellwig *et al.*, 1997; Thomas *et al.*, 1986), and DINP (Hellwig *et al.*, 1997) were negative or only induced a low incidence of malformations at maternally toxic dosage levels. Dosage levels above those used in the current study (1–4 g/kg/day) (Ema *et al.*, 1992, 1993, 1994; Hellwig *et al.*, 1997; Thomas *et al.*, 1986). It is apparent from a review of the standard teratology studies that this test is inadequate for risk assessment of endocrine-disrupting chemicals. Endocrine-disrupting chemicals like the PEs, vinclozolin, procymidone, p,p′ DDE, and linuron are false negatives in this assay. The inadequacy of this protocol arises from several sources. In older teratology studies the exposure period in the rat (from GD 6 to GD 15) did not include the critical period of sexual differentiation (Gray *et al.*, 1994, 1999a,b; Wolf *et al.*, 2000). Even studies conducted under the new developmental toxicity guidelines with the exposure continued through GD 19 (which includes some, but not all, of the period of sexual differentiation) cannot identify many of the malformations in male pups. Most reproductive malformations, induced *in utero*, are latent and are not apparent until after puberty or later in life (hypospadias, retained nipples, agenesis of sex accessory glands, prostatitis, and reproductive senescence). For example, in contrast to the results seen here with BBP, which induced malformations in 84% of the male offspring, administration of 2% BBP in the diet from GD 16–20 was found to be without effect on the fetal rat (Ema *et al.*, 1992).

The multigenerational study design is currently the only test protocol that provides an adequate exposure regime and an examination of the reproductive function of the offspring. However, multigenerational studies that were conducted prior to the new U.S. EPA guidelines occasionally failed to detect malformations produced by chemicals with antiandrogenic activity, as the assessment of the reproductive system of the F1 was not thorough enough (Hodge *et al.*, 1968; Waterman *et al.*, 2000). In this regard, the new U.S. EPA reproductive test guidelines are a considerable improvement, as they have included several end points sensitive to endocrine disruption and require that three F1 animals per sex per litter be examined for gross malformations at weaning. However, it is unclear if the examination of weanling animals for reproductive malformations is entirely adequate, because the reproductive tissues are immature and some of them are quite small at this time. One may be able to detect severe hypospadias at weaning, but it is likely that many effects such as epispadias or agenesis of the sex accessory glands would not be detected at weaning.

For the PEs, few published multigenerational studies exist, even though these are high-production-volume chemicals that have been used for several decades. Multigenerational or transgenerational studies on DBP have been conducted in three laboratories using rats (Gray *et al.*, 1999a; Mylchreest *et al.*, 1998, 1999, 2000; Wine *et al.*, 1997). These studies report similar reproductive effects in the F1 males with LOAELs of 66–100 mg/kg/day. F1 females were affected at 250 mg DBP/kg/day (Gray *et al.*, 1999a), while adverse effects on the P0 females and males also were noted at 500 and 1000 mg DBP/kg/day (Gray *et al.*, 1999a). DBP, DEHP, and di-n-hexyl phthalate also produced adverse reproductive effects in the continuous breeding protocol, with DEHP affecting both male and female mice (Lamb *et al.*, 1987).

In contrast to the effects of DINP seen in the current study, a two-generation study of DINP in the diet at doses up about 500 mg/kg/day reported no adverse reproductive effects including fertility, testis weight, or histology in the F1 generation (Nikiforov *et al.*, 1996; Waterman *et al.*, 2000). However, this study did not include all of the androgen-sensitive end points measured herein, or examine a sufficient number of F1 animals to detect the low (7.7%) incidence of malformations that we found. Among other end points, they did not measure permanent nipples, areolas in infants, preputial separation, testis or epididymal sperm counts, or weigh androgen-dependent organs like the levator ani. In addition, neither the numbers of testes and epididymides examined histologically nor the histological results were presented, so it is unclear how many tissues were examined or what lesions, if any, were observed. Recently, we found that administration of DINP from GD 14 to

PND 3 at doses of 1 and 1.5 g/kg/day reduced AGD (at 1.5) and increased the incidence of areolas in a dose-related fashion (L. E. Gray, unpublished data), confirming the weak antiandrogenic activity of DINP reported herein. Not unexpectedly, developmental toxicity studies with DINP are largely negative. In one developmental toxicity study of DINP, doses up to 1000 mg/kg/day (GD 6–15) were not teratogenic or selective developmental toxicants (Waterman *et al.*, 1999). Another study found that the prenatal toxicity of DINP varied from mixture to mixture (their DINP with the same CAS number as used herein did not cause reproductive malformations), with only a few reproductive malformations seen at 1000 mg/kg/day [gonads in abnormal position in two fetuses (Hellwig *et al.*, 1997)]. Herein, using end points sensitive to androgen disruption during fetal life, we found that DINP was antiandrogenic, inducing areolas and malformations (4/52) in male offspring, effects that could not possibly be assessed in these two teratology studies.

Relative Potency of PEs (PE-TEFs)

Although the current study only used a single dose level of each PE (the doses are low compared to those used in most developmental toxicity and pubertal studies of testicular toxicity), we are still able to make a preliminary estimate of the relative potency of PEs. Until recently, risk assessments were conducted on a chemical-by-chemical basis. However, as mandated under the Food Quality Protection Act (FQPA) of 1996, risk assessors are beginning to consider the risk posed by combined exposure to chemicals that act via the same mechanism. Therefore, risk assessments for PE-induced reproductive toxicity should consider these chemicals as a group and include exposures from multiple sources. Once adequate dose-response studies have been conducted, phthalate ester toxic equivalence factors (PE-TEF) can be developed for reproductive toxicity induced *in utero*. We propose the following preliminary PE-TEFs as follows; DEHP-TEF = 1; BBP-TEF = 1; DBP-TEF = 0.5 (based on the work of Mylchreest *et al.*, 1999; Gray *et al.*, 1999a); DINP (for the CAS # and lot used herein only)-TEF = 0.05–0.1; DOTP-, DMP-, AND DEP-TEF = 0. These values are presented to stimulate discussion and research about how we should estimate cumulative and aggregate risk to PEs. The PE-TEFs presented here, obviously, will be refined upon completion of adequate dose-response evaluations of the transgenerational effects of PEs.

DEP, DMP, and DOTP did not alter male rat sexual differentiation in the current study. Hence, the SAR profile for developmental reproductive toxicity of the PEs resembles that seen in young male rats (Foster *et al.*, 1980), implying that there may be some similarity in the mechanism of action of the PEs *in utero* and during puberty, although the effects are very different. It appears that PEs with monoester metabolites with an ester group 4–6 carbons long are developmental reproductive toxicants. In order for a phthalate diester to be metabolized to an active monoester, the ester groups must be in the ortho position. DOTP, which is isomeric with DEHP but reportedly not metabolized to MEHP, was inactive in the current study and failed to cause testicular lesions in young adult Sprague-Dawley rats (administered as 1% in the diet for 90 days, with treatment initiated at 8 weeks of age; Barber and Topping, 1995).

As the numbers of litters and pups in the DEP and DMP groups are smaller than the other groups, we pooled the data from these two PEs for statistical purposes, given that these are both short- chain esters and we anticipated no effect from either. When the data were examined in this manner, the values for the DEP plus DMP (7 litters and 32 males) male offspring did not differ from controls. Our confidence in the DEP and DMP data are high, as these PEs did not induce areolas as did DINP, which was active but relatively weak as compared to BBP and DEHP. Nevertheless, as high levels of MEP, the monoester of DEP, have been detected in human urine (Brock *et al.*, 2000), we plan to reexamine this PE to substantiate the lack of effect reported here.

Species-Specific PE-Induced Testicular Toxicity?

Koop and Juberg (1999) dismissed the reproductive and developmental effects of the PEs as irrelevant due to a species-specific peroxisome proliferator–activated receptor α (PPARα) mechanism, with effects occurring only in a few strains of rats and mice. However, a thorough review of the literature indicates otherwise. First, the testicular toxicity of the PEs appears to be unrelated to the species-specific expression of PPARα, as evidenced by the display of testicular pathology in DEHP-treated PPARα knockout mice (Ward *et al.*, 1998). Second, a broad range of vertebrate species display testicular toxicity after PE treatment during development. The PEs have been shown to cause testicular alterations in numerous mammalian species, as long as the exposure included *in utero* or pubertal development. Affected vertebrates include several strains of rat (Long Evans, Sprague-Dawley, and Wistar), mice (including PPARα knockouts; Ward *et al.*, 1998), hamsters (severe seminiferous tubular atrophy in most males after MEHP-treatment; Gray *et al.*, 1982), ferrets (Lake *et al.*, 1976), guinea pigs (Gray *et al.*, 1982), and rabbits (Higuchi *et al.*, 1999). In addition to mammals, fish (Patyna *et al.*, 1999) and frogs (Higuchi *et al.*, 1999) also display adverse reproductive outcomes when exposed to PEs during development. In fact, to date there are no studies of DEHP or MEHP that display negative results when the dosing regime includes the perinatal or pubertal periods of life and a sufficient number of animals is adequately evaluated. A study of DEHP in the nonhuman primate, which dosed four adult male common marmosets/group with DEHP at 100, 500, and 2500 mg/kg for 13 weeks, did not observe any testicular effects (Kurata *et al.*, 1998). However, fetal and prepubertal nonhuman primates have not been similarly evaluated. Even in the rat, a species sensitive to PE-induced testicular damage during fetal and pubertal life, the testis is much less sensitive to the effects of PEs during adult life.

PE Exposures in Humans and Rats as Related to the Current Study

Although the current focus is on PEs in toys, these chemicals are ubiquitous in the environment. Some groups have particularly high DEHP exposures, with serum levels of MEHP in the ppm range (i.e., dialysis patients, transfusions, and occupational exposures). In addition, it was recently reported that human urine contained surprisingly high (ppm levels) of phthalate monoesters from as yet unidentified sources (Blount *et al.*, 2000). A study of the exposure of newborn infants to DEHP and MEHP resulting from transfusions found plasma levels of DEHP between 3.4 and 11.1 μg/ml, while MEHP ranged from 2.4 to 15.1 μg/ml (Sjoberg *et al.*, 1985a). Plonait *et al.* (1993) observed similar levels of DEHP in the serum of newborn infants after transfusion (6.1–21.6 μg DEHP/ml serum). For comparison, in male rats the concentrations of DEHP and MEHP in the serum 3 h after a single oral dose of 2.8 g/kg, which induces testicular lesions, are only about 4-fold higher than the levels in infants on dialysis, being 8.8 ± 1.7 and 63.2 ± 8.7 μg/ml, respectively (Teirlynck and Belpaire, 1985). In another study, Sjoberg *et al.* (1985b) reported that the testicular damage caused by treatment with 1 g DEHP/kg for 14 days to 25-day-old rats (40- and 60-day-old rats were unaffected) was associated with concentrations of MEHP in plasma ranging from 48 to 152 μg/ml. As the dosing regime used by Sjoberg *et al.* (1985b) is similar to that employed in the current study (0.75 g/kg/day for 12 days), which induced malformations in more than 80% of the male offspring, we would predict that maternal serum levels of MEHP in the current study would be about 35–115 μg/ml, which would be about 10-fold higher than the levels seen in dialysis patients. Assuming that administered dose is proportional to serum MEHP levels in the rat, we also would expect serum MEHP levels in the rat at the LOAEL (37 mg/kg/day) and NOAEL would be equivalent to those seen in dialysis patients. It is evident that dose-response studies that examine the effects of exposure to low levels of PEs are required and that these studies also should examine target tissue levels of DEHP and MEHP so they can be linked to known human exposure scenarios. Future animal studies should also include a sufficiently broad range of androgen-sensitive responses and use a sufficient number of animals such that malformations like those seen here in the DINP group (7.7% malformed) can be statistically detected.

Conclusions

The PEs DEHP, DBP, BBP, and DINP alter male rat sexual differentiation in an antiandrogenic fashion. While the specific mechanism of action remains to be identified, PE-induced reductions in fetal T levels result in malformations of androgen-dependent tissues. The structure-activity relationship for these fetal effects resembles that for pubertal testicular toxicity, implying that similar cellular and molecular targets are involved and that this target remains susceptible until fully differentiated after puberty, when reproductive effects can no longer be easily induced. Given that PE-induced reproductive lesions are displayed by a wide range of vertebrate species and the role of androgens and steroid hormone synthesis are highly conserved throughout the class Mammalia, it is premature to conclude that PE-induced alterations of sexual differentiation would also not be induced in the human if the male was exposed to concentrations of active PE metabolites during critical stages of reproductive development. Until we are certain of the precise mechanism responsible for PE-induced alteration of testicular function in the rat, it is impossible to accurately define the critical window of susceptibility in humans.

ACKNOWLEDGMENTS

We would like to thank Christy Lambright, Cynthia Wolf and Carol Moeller for their excellent technical assistance in this project.

REFERENCES

Adams, N. R. (1989). Phytoestrogens. In *Toxicants of Plant Origin* (P. R. Cheeke, Ed.). Phenolics, Vol. 4, pp. 23–51. CRC Press, Boca Raton.

Ankley, G. T., and Giesy, J. P. (1998). Endocrine disruptors in wildlife: a weight of evidence perspective. In *Principles and Processes for Evaluating Endocrine Disruption in Wildlife* (R. Kendall, R. Dickerson, J. Giesy, and W. Suk, Eds.), pp. 349–367. SETAC Press, Pensacola.

Arcadi, F. A., Costa, C., Impertore, C., Marchese, A., Rapisarda, A., Salemi, M., Trimarchi, G. R., and Costa, G. (1998). Oral toxicity of bis(2-ethylhexyl) phthalate during pregnancy and suckling in the Long-Evans rat. *Food Chem. Toxicol.* **36,** 963–970.

Ashby, J., Tinwell, H., Lefevre, P., Odum, J., Paton, D., Milward, S., Tittensor, S., and Brooks, A. N. (1997). Normal sexual development of rats exposed to butyl benzyl phthalate from conception to weaning. *Regul. Toxicol. Pharmacol.* **26,** 102–118.

Barber E. D., and Topping, D. C. (1995). Subchronic 90-day oral toxicology of di(2-ethylhexyl) terephthalate in the rat. *Food Chem. Toxicol.* **33,** 971–978.

Blount, C. B., Silva, M. J., Caudill, S. P., Needham, L. L., Pirkle, J. L., Sampson, E. J., Lucier, G. W., Jackson, R. J., and Brock, J. W. (2000). Levels of seven urinary phthalate metabolites in a human reference population. *Environ. Health Perspect.* **108,** 979–982.

Cain, J. C. (1960). Miroestrol: an oestrogen from the plant *Pueraria mirifica*. *Nature* **188,** 774–777.

Carlsen, E., Giwercman, A., Keiding, N., and Skakkebaek, N. E. (1992). Evidence for decreasing quality of semen during past 50 years. *Br. Med. J.* **305,** 609–613.

Colborn, T., and Clement, C. (1992). *Advances in Modern Environmental Toxicology.* Vol. XXI. Chemically induced alterations in sexual and functional development: the wildlife/human connection. Princeton Scientific Publishing Co. Inc., Princeton, NJ.

EC 11/98 Opinion on Phthalate Migration (1998). Available at: http://europa.eu.int/comm/food/fs/sc/sct/out19_en.html. Accessed October 11, 2000.

EC (1999). Proposed ban on several phthalates. Available at: http://europa.eu.int/comm/dg03/press/1999/IP99829.html. Accessed October 11, 2000.

Ema, M., Itami, T., and Kawasaki, H. (1992). Effect of period of exposure on the developmental toxicity of butyl benzyl phthalate in rats. *J. Appl. Toxicol.* **12,** 57–61.

Ema, M., Amano, H., Itami, T., and Kawasaki, H. (1993). Teratogenic evaluation of di-n-butyl phthalate in rats. *Toxicol. Lett.* **69,** 197–203.

Ema, M., Amano, H., and Ogawa, Y. (1994). Characterization of the developmental toxicity of di-n-butyl phthalate in rats. *Toxicology* **86,** 163–174.

Foster, P. M., Mylchreest, E., Wallace, D. G., and Sar, M. (1999). Antiandrogenic effects of phthalate esters on male reproductive development in the rat. Abstract S5.3, European Teratology Society, Oxford, UK.

Foster, P. M., Thomas, L., Cook, M., and Gangolli, S. (1980). Study of the testicular effects and changes in zinc excretion produced by some n-alkyl phthalates in the rat. *Toxicol. Appl. Pharmacol.* **54,** 392–398.

Giesy, J. P., and Snyder, E. M. (1998). Xenobiotic modulation of endocrine function in fishes. In *Principles and Processes for Evaluating Endocrine Disruption in Wildlife* (R. Kendall, R. Dickerson, J. Giesy, and W. Suk, Eds.), pp. 155–237. SETAC Press, Pensacola.

Gray, L. E., Jr. (1991). Delayed reproductive effects following exposure to toxic chemicals during critical developmental periods. In *Aging and Toxicology: Biological and Behavioral Perspectives* (R. L. Cooper, J. M. Goldman, and T. J. Harbin, Eds.), pp. 183–210. Johns Hopkins University Press, Baltimore, MD.

Gray, L. E., Jr. (1992). Chemical-induced alterations of sexual differentiation: a review of effects in humans and rodents. In *Advances in Modern Environmental Toxicology* (T. Colborn and C. Clement, Eds.), Vol. 21, pp. 203–230. Princeton Scientific Publishing Co. Inc., Princeton, NJ.

Gray, L. E., Ostby, J., and Kelce, W. R. (1994). Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rats. *Toxicol. Appl. Pharmacol.* **129,** 46–52.

Gray, L. E., Jr., Wolf, C., Lambright, C., Mann, P., Price, M., Cooper, R., and Ostby, J. (1999a). Administration of the potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p′-DDE and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol. Ind. Health* **15,** 94–118.

Gray, L. E., Jr., Ostby, J., Monosson, E., and Kelce, W. R. (1999b). Environmental antiandrogens: low doses of the fungicide vinclozolin alter sexual differentiation of the male rat. *Toxicol. Ind. Health* **15,** 48–64.

Gray, T. J. B., Rowland, I. R., Foster, P. M. D., and Gangolli, S. D. (1982). Species differences in the testicular toxicity of phthalate esters. *Toxicol. Lett.* **11,** 141–147.

Hellwig, J., Freudenberger, J., and Jackh, R. (1997). Differential prenatal toxicity of branched phthalate esters in rats. *Food Chem. Toxicol.* **35,** 501–512.

Higuchi, T., Kane, C., Palmer, J., and Veeramachaneni, D. N. R. (1999). Developmental effects of DBP in frogs and rabbits. *Biol. Reprod.* **60**(suppl 1), p 153.

Hodge, H. C., Downs, W. L., Smith, D. W., Maynard, E. A., Clayton, J. W., Jr., and Pease, H. L. (1968). Oral toxicity of linuron (3-(3,4-dichlorophenyl-1-methoxy-1-methylurea) in rats and dogs. *Fundam. Cosmetol. Toxicol.* **6,** 171–183.

Imperato-McGinley, J., Sanchez, R. S., Spencer, J. R., Yee, B., and Vaughan, E. D. (1992). Comparison of the effects of the 5 alpha-reductase inhibitor finasteride and the antiandrogen flutamide on prostate and genital differentiation: dose-response studies. *Endocrinology* **131,** 1149–1156.

Kelce, W. R., Monosson, E., Gamcsik, M. P., Laws, S., Gray, L. E., Jr. (1994). Environmental hormone disruptors: evidence that vinclozolin developmental toxicity is mediated by antiandrogenic metabolites. *Toxicol. Appl. Pharmacol.* **126,** 276–285.

Koop, C. E., and Juberg, D. R. (1999). Review and Consensus Statement. A scientific evaluation of the health effects of two plasticizers used in medical devices and toys: a report from the American Council on Science and Health. *Medscape Gen. Med. J.,* June 22, 1–35.

Kurata, Y., Kidachi, F., Yokoyama, M., Toyota, N., Tsuchiani, M., and Katoli, M. (1998). Subchronic toxicity of di(2-ethylhexyl)phthalate in common marmosets: lack of hepatic peroxisome proliferation, testicular atrophy, or pancreatic acinar cell hyperplasia. *Toxicol. Sci.* **42,** 49–56.

Lake, B., Brantom, P., Gangolli, S., Butterworth, K. R., and Grasso, P. (1976). Studies on the effects of orally administered di-(2-ethylhexyl)-phthalate in the ferret. *Toxicology* **6,** 341–356.

Lamb, J., IV, Chapin, R., Teague, J., Lawton, A. D., and Reel, J. R. (1987). Reproductive effects of four phthalate acid esters in the mouse. *Toxicol. Appl. Pharmacol.* **88,** 255–269.

Monosson, E. (1997). Reproductive and developmental effects of contaminants in fish populations: establishing cause and effect. In *Chemically Induced Alterations in Functional Development and Reproduction in Fishes* (R. Rolland, M. Gilbertson, and R. E. Peterson, Eds.), pp. 177–194. SETAC Press, Pensacola.

Mylchreest, E., Cattley, R., and Foster, P. (1998). Male reproductive tract malformations in rats following gestational and lactational exposure to di-(n-butyl)-phthalate: an antiandrogenic mechanism? *Toxicol. Sci.* **43,** 47–60.

Mylchreest, E., Sar, M., Cattley, C., and Foster, P. M. (1999). Disruption of androgen-regulated male reproductive development by di-(n-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol. Appl Pharmacol.* **156,** 81–95.

Mylchreest, E., Wallace, D. G., Cattley, R. C., Foster, P. M. (2000). Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to di(n-butyl) phthalate during late gestation. *Toxicol. Sci.* **55,** 143–51.

Nikiforov, A. I., Keller, L. H., and Harris, S. B. (1996). Lack of transgenerational reproductive effects following treatment with di-isononyl phthalate. *Toxicologist* **30,** 119.

Ostby, J., Kelce, W. R., Lambright, C. S., Wolf, C. J., Mann, P., and Gray, L. E., Jr. (1999). The fungicide procymidone alters sexual differentiation in the male rat by acting as an androgen-receptor antagonist *in vivo* and *in vitro*. *Toxicol. Ind. Health* **15,** 80–93.

Parks, L. G., Ostby, J. S., Lambright, C. R., Abbott, B. D., and Gray, L. E., Jr. (1999). Perinatal butyl benzyl phthalate (BBP) and di-(2-ethylhexyl) phthalate (DEHP)exposures induce antiandrogenic effects in Sprague-Dawley (SD) rats. *Biol. Reprod.* **60**(Suppl 1), 153.

Parks, L. G., Ostby, J. S., Lambright, C. R., Abbott, B. D., Klinefelter, G. R., Barlow, N.J., and Gray, L. E., Jr. The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. *Toxicol. Sci.* **58,** 339–349.

Patyna, P., Thomas, P., and Cooper, K. (1999). Multigenerational reproductive effects of DBP in Japanese medaka (*Oryzias latipes*). *Toxicologist* **48,** 262.

Plonait, S., Nau, H., Maier, R., Wittfoht, W., Obladen, M. (1993). Exposure of newborn infants to di-(2-ethylhexyl)-phthalate and 2-ethylhexanoic acid following exchange transfusion with polyvinylchloride catheters. *Transfusion* **33,** 598–605.

Poon, R., Lecavalier, P., Mueller, R., Valli, V., Procter, B., and Chu, I. (1997). Subchronic oral toxicity of di-n-octyl phthalate and di(2-ethylhexyl) phthalate in the rat. *Food Chem. Toxicol.* **35,** 225–239.

Schardein, J. L. (1993). Hormones and hormonal antagonists. In *Chemically Induced Birth Defects.* pp. 271–339. Marcel Dekker, New York.

Sharpe, R. M., and Skakkebaek, N. E. (1993). Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? *Lancet* **341,** 1392–1395.

Sharpe, R. M., Fisher, J. S., Millar, M., Jobling, S., and Sumpter, J. (1995). Gestational and lactational exposure to xenoestrogens results in reduced testicular size and sperm production. *Environ. Health Perspect.* **103,** 1136–1143.

Sjoberg, P. O., Bondesson, U. G., Sedin, E. G., and Gustafsson, J. P. (1985a).

Exposure of newborn infants to plasticizers. Plasma levels of di-(2-ethylhexyl) phthalate and mono-(2-ethylhexyl) phthalate during exchange transfusion. *Transfusion* **25,** 424–428.

Sjoberg, P., Bondesson, U., Kjellen, L., Lindquist, N. G., Montin, G., and Ploen, L. (1985b). Kinetics of di-(2-ethylhexyl) phthalate in immature and mature rats and effects on testis. *Acta Pharmacol. Toxicol.* **56,** 30–37.

Teirlynck, O. A., and Belpaire, F. (1985). Disposition of orally administered di-(2-ethylhexyl) phthalate and mono-(2-ethylhexyl) phthalate in the rat. *Arch. Toxicol.* **57,** 226–230.

Thomas, J., Wienchkowski, D. B., Gilles, B. A., Thomas, M. J., and Youkilis, E. J. (1986). Effects of phthalic acid esters on the neonate and aspects of teratogenic actions. *Environ. Health Perspect.* **65,** 243–248.

Ueno, Y. (1985). The toxicology of mycotoxins. *Crit. Rev. Toxicol.* **14,** 99–132.

Van Der Kraak, G, Munkittrick, K. R., McMaster, M. E., and MacLatchy, D. (1998). A comparison of bleached kraft mill effluent, 17β estradiol, and β sitosterol effects on reproduction in fish. In *Principles and Processes for Evaluating Endocrine Disruption in Wildlife.* (R. Kendall, R. Dickerson, J. Giesy, and W. Suk, Eds.), pp. 294–265. SETAC Press, Pensacola, FL.

Ward, J. M., Peters, J. M., Perella, C. M., and Gonzalez, F. J. (1998). Receptor and nonreceptor-mediated organ-specific toxicity of di-(2-ethylhexyl) phthalate in peroxisome proliferator-activated receptor-alpha-null mice. *Toxicol. Pathol.* **26,** 240–246.

Waterman, S. J., Ambroso, J. L., Keller, L. H., Trimmer, G. W., Nikiforov, A. I., and Harris, S. B. (1999). Developmental toxicity of di-isodecyl and di-isononyl phthalates in rats. *Reprod. Toxicol.* **13,** 131–136.

Waterman S. J., Keller L. H., Trimmer, G. W., Freeman, J. J., Nikiforov, A. I., Harris, S. B., Nicolich, M. J., and McKee, R. H. (2000). Two-generation reproduction study in rats given di-isononyl phthalate in the diet. *Reprod. Toxicol.* **14,** 21–36.

Wilson, J. D. (1978). Sexual differentiation. *Ann. Rev. Physiol.* **40,** 279–306.

Wine, R. N., Li, L. H., Barnes, L., Gulati, D. K., and Chapin, R. E. (1997). Reproductive toxicity of di-n-butylphthalate in a continuous breeding protocol in Sprague-Dawley rats. *Environ. Health Perspect.* **105,** 102–107.

Wolf, C. J., Ostby, J., and Gray, L. E., Jr. (2000). Characterization of the period of sensitivity of fetal male sexual development to vinclozolin. *Toxicol. Sci.* **55,** 152–161.

Aveda Case Study

CASE STUDY

Aveda: Optimizing Post-Consumer Content and Beyond

How does a company engaged in the fiercely competitive health and beauty products industry move beyond paying lip service to environmental concerns? At Minneapolis-based Aveda, which has developed one of the world's most enlightened packaging programs, it starts with the commitment of founder Horst Rechelbacher and president Dominique Conseil. Along with upper management, they encourage employees to continually improve the environmental performance of both their products and their packaging. Granted, Aveda (a $500 million autonomous subsidiary of the Esteé Lauder corporation) faces the challenge of being a global company that sources, manufactures, and ships products from continent to continent. This means that any gains in environmental performance must be viewed against the backdrop of international transportation impacts, packaging materials that need to be large enough to accommodate multiple languages, raw material sourcing policies, and other concerns. Still—in a business where image is everything—Aveda uniquely surfaces as a company that proactively ranks environmental packaging goals above short-term pricing and sometimes even aesthetic considerations. Any company interested in serious packaging reform can benefit from a study of their policies and achievements.

Breaking the Mold

Optimization best describes Aveda's approach to packaging design. For nearly a decade, the company has been dematerializing its packaging—by lightweighting containers as well as by maximizing the post-consumer recycled (PCR) content of plastics such as high-density polyethylene (HDPE), polypropylene, glass, and other materials. According to Mary Tkach, Aveda's director of environmental sustainability, product and packaging designers interact closely in order to find ways to continually increase the post-consumer content in all aspects of packaging, from primary bottles and squeezable tubes to secondary paperboard boxes and collateral materials.[84] Even their pallets and transport packaging have been addressed.

"Solutions can range from reformulating the product in a package to changing the packaging itself," says Tkach. A product might be made more liquid, so it could be bottled in a high PCR content HDPE container rather than a squeezable tube. One hundred percent PCR paperboard cover-stock paper might be specified for sleeves and cartons on some products. Or the secondary packaging could be eliminated altogether. In certain cases, the product could be modified. The natural essential oils that form the bases for most of the company's products, for example, are more active than synthetic oils. This has required careful testing and collaboration with suppliers to ensure that the package and the product are compatible.

Such work is time consuming. One critical obstacle can be finding suppliers who have the technology and willingness to push the envelope of post-consumer content. At times Aveda has accepted an increase in material costs in order to achieve its environmental goals, such as a rare 16 percent price increase to maximize the PCR content of an extruded tube package. Post-consumer materials haven't always commanded a premium, however, especially after the research and development has been completed. In fact, using high PCR content can be money saving despite a system heavily skewed toward virgin resource extraction. A 10-year effort to redesign shampoo bottles to make them as thin as possible and maximize the post-consumer resin content now saves the company about $1 million per year.[85]

Design Priorities

John Delfausse, vice president of packaging development, explains that Aveda's approach to maximizing post-consumer plastics content has evolved with experience, which, in turn, has informed a hierarchy of design priorities.[86] Aveda now actually ranks environmental performance ahead of cost and design concerns, even though all three are essential packaging considerations. "The first question designers ask is whether the material is recyclable," explains Delfausse. "A second priority is to build component parts out of a single material so that they can easily be sorted and recycled."

Plastics Hierarchy

The following illustrates Aveda's hierarchy when considering the use of plastic for packaging.

Most preferred:
High-Density Polyethylene (HDPE)
Low-Density Polyethylene (LDPE)

Acceptable:
Polyethylene Terephthalate (PET)
Ethylene Vinyl Acetate (EVA)
Polypropylene (PP)

Least Preferred:
Polyurethanes (PU)
Polystyrene (PS)
Acrylonitrile Butadiene Styrene (ABS)
Polycarbonates (PC)
Acrylic

Prohibited:
Polyvinyl Chloride (PVC)

Source: Aveda Corp. Material User Manual, as cited in "Wrap Artists: How Aveda Bundles Sustainability into Its Packaging," The Green Business Letter, December 2003, p. 5.

An official *Material User Manual* has been developed to guide departments through the optimization process. Aveda's "most preferred" resins are high- and low-density polyethylene (HDPE and LDPE). Polypropylene is an "acceptable" material, also favored by Aveda packaging designers. All three have fairly clean supply sources, consume comparatively less energy in manufacturing, and contain little or no toxic or carcinogenic materials. Even though it is highly recyclable, polyethylene teraphthalate (PET) is used only occasionally. It is avoided whenever possible because of the "bad actors" it contains such as antimony, a plasticizer used in the production process; when they do choose PET, designers specify the maximum post-consumer content possible. Out of the desire to avoid hazardous substances, the company has stopped using polyvinyl chloride (PVC) packaging altogether. (Esteé Lauder, Aveda's parent corporation, has taken a similar pledge.)

Achieving such high rates of single-material, post-consumer content has been a methodical process for Aveda. Concerned both about unintended interactions between the essential oils in its natural products that contain post-consumer plastics and about the printability and aesthetics of containers, Aveda originally sandwiched "junk" material between inner and outer layers of virgin plastic. Company designers worked continually with suppliers to increase post-consumer content incrementally, first with the outer printable layer, and later with the inner surface. Satisfied that maximizing recycled content was a totally acceptable and even desirable solution, Aveda now boasts the highest levels of post-consumer content packaging in its industry. Two decades ago nearly all HDPE was derived from virgin materials, but Aveda has succeeded in raising the PCR content in much of its packaging to between 80 and 100 percent, with very little containing less than 50 percent. Aveda has worked with 100 percent post-consumer newsprint molded pulp, 94 percent PCR glass, and even agricultural crop residues—the "shives" or straw from flax—blended with plastic resins for a lipstick container.

A Systems Approach

Caps, spray mechanisms, and multicomponent parts can be the bane of any environmental packaging designer who is attempting against all odds to fashion the ideal package made out of single, easily recyclable material. For that reason, Delfausse puts a great deal of thought into systems that might facilitate collection, or packaging "take back" or "leasing."

"Aveda needs upwards of 20 million caps per year," reasons Delfausse. "That's a lot of PCR material. A fairly simple infrastructure could be set up within our 200 retail stores and 6,000 salons to collect the caps. Unfortunately, in the past when we've tried this we've ended up with a lot of garbage." Still, Delfausse hopes to introduce a cap recovery

system sometime in 2005 and is investigating the possibilities of in-store refill stations as well.

In addition to its own internal design guidelines, Aveda relies on a software program called Merge for further qualitative analysis. Developed by the Boston-based Alliance for Environmental Innovation, a project of Environmental Defense, Merge scores and rates products and packages on a series of metrics: packaging resource consumption; packaging energy consumption; virgin materials content; nonrecyclable materials content; presence of known toxins; greenhouse gases; and pallet efficiency.

"Balancing the costs and trade-offs of packaging can be extremely complex," says Tkach. "Is it better to use a 50 PCR/50 virgin (chlorine-bleached) paperboard or to use a 20 PCR/80 virgin (non-chlorine-bleached) paperboard? The key is having good information and a framework to analyze those options."

That the company has succeeded in achieving such high rates of PCR content without compromising the product should send a clear message to the rest of the industry. Post-consumer plastic, however, is not necessarily transparent. One of the challenges of designing packages with high PCR content is adjusting to a minimal graying in color. "Everybody from the president on down had to accept that the bottles' color turned slightly grayer," says Delfausse. "But by doing it, we save about 150 tons of virgin polyethylene on an annualized basis."

Beyond Confidentiality

While occasionally compromising on higher materials costs and accepting a slightly abbreviated color palette may seem questionable practices for many corporate capitalists, it is Aveda's stand on confidentiality that is perhaps most unusual. The company doesn't view its hard-won environmental R&D discoveries as proprietary trade secrets. Instead, it has gone out of its way to advertise them, sponsoring meetings with suppliers and sharing material research with numerous injection molders. Favored suppliers have frequently been revealed in articles and Web site reports—Johnson Printing for cartons and sleeves; ALCAN Packaging and Owens Illinois for bottles; CCL Plastics for tubes; TricorBraun and Kaufman Container for bottles, jars, and caps. A few manufacturers have taken notice and made the switch.

"One of our goals is to enable other companies to carry on with the work of these new materials," says Delfausse. "The sooner others can get started, the better off everyone will be."

Plastic Additives to Research
Feel free to also select your own plastic additive, just make sure that you look at one specific plastic additive, and not a more general group of plastic additives. You may also select your plastic additive from the list below:

Phthalates (pick one)
- DEHP (di-2-ethylhexyl phthalate)
- DEP (diethyl phthalate)
- DBP (dibutyl phthalate)
- BzBP (benzylbutyl phthalate)
- etc

Bisphenol-A
Bromiated Flame Retardants (pick one)
- PBB (polybrominated biphenyl)
- PBDE (polybrominated diphenyl ether)
- HBCD (hexabromocyclododecane)
- TBBPA (tetrabromo-bisphenol-A)
- etc

Triclosan

Assignment
Your goal is to find several summaries of information on your plastic additive, as well as several studies. You will then present your findings to the rest of the class. See rubric for specific requirements.

Websites for background information (make note of the biases!)
www.ewg.org a decidedly environmentally leaning website
www.fda.gov
www.niehs.nih.gov
www.hhs.gov
www.americanchemistry.com an industry source

For studies, search using Google Scholar.

RUBRIC- PLASTIC ADDITIVE PRESENTATIONS

	Below Satisfactory	Max pts	Satisfactory	Max pts	Excellent	Max pts
Summaries of 5 studies (at least 3 human if possible)	Incorrect information, under 5 studies	10	Summaries are brief, but don't fully describe the studies. Only one type of study was examined.	30	Brief summaries of 5 studies that concisely sum up the results and conclusions of the studies. A wide range of studies was used, covering both animal and human studies.	40
General overview of current	Incorrect information, overview is	10	Overview of current concerns lacks either two perspectives or the	30	Overview of current concerns includes *at least* two perspectives, and	40

concerns	lacking		current regulations in effect, but is otherwise thorough (if 2nd perspective is lacking, the source of the one perspective must be *clearly* stated)		states the current regulations in effect (if any)	
Overall Clarity of Presentation	Presentation was difficult to follow	5	Some areas of the presentation were unclear, or time limit was exceeded	10	Presentation was concise, within the time limit and included clear explanations.	20
Total						

RUBRIC- PLASTIC ADDITIVE PRESENTATIONS

	Below Satisfactory	Max pts	Satisfactory	Max pts	Excellent	Max pts
Summaries of 5 studies (at least 3 human if possible)	Incorrect information, under 5 studies	10	Summaries are brief, but don't fully describe the studies. Only one type of study was examined.	30	Brief summaries of 5 studies that concisely sum up the results and conclusions of the studies. A wide range of studies was used, covering both animal and human studies.	40
General overview of current concerns	Incorrect information, overview is lacking	10	Overview of current concerns lacks either two perspectives or the current regulations in effect, but is otherwise thorough (if 2nd perspective is lacking, the source of the one perspective must be *clearly* stated)	30	Overview of current concerns includes *at least* two perspectives, and states the current regulations in effect (if any)	40
Overall Clarity of Presentation	Presentation was difficult to follow	5	Some areas of the presentation were unclear, or time limit was exceeded	10	Presentation was concise, within the time limit and included clear explanations.	20
Total						