A Testing Deadline for Endocrine Disrupters

EPA scrambles to develop a screening program for these complex substances.

MARGIE PATLAK

In reaction to growing evidence that environmental pollutants that interfere with the endocrine system can harm the health of humans and wildlife species, Congress this past summer gave EPA a tight timetable for action. Within two years, the agency must develop a strategy to screen and test common chemicals for endocrine disrupter effects, and the program must be implemented just one year after that.

EPA has already begun to decide which of the nearly 80,000 commercial chemicals should be tested for hormonal activity. According to Lynn Goldman, assistant administrator of EPA's Office of Prevention, Pesticides, and Toxic Substances, her office has already whittled that number down to about 17,000 common chemicals that are in commerce at high volume. She plans to further shrink this number by focusing on chemicals found in drinking water, food, ambient air, or household products. EPA researchers are also scrutinizing the three-dimensional structures of known endocrine disrupters to look for similar traits that can be used to predict which groups of chemicals are likely to have hormonal effects.

But researchers and regulators scrambling to meet the deadline are discovering that endocrine disrupters pose a unique set of challenges. The effects of these compounds can vary, depending on which tissues are tested, when during an organism's development the compounds are administered, and whether other environmental estrogens are present. Some effects may not surface for more than 25 years and, unlike most toxic substances, the greatest effects of endocrine disrupters are often seen with some of the smallest doses tested. Because endocrine disrupters can so aptly mimic natural hormones, it is often hard to separate their effects from those of hormones generated by the body.

Researchers are just beginning to tackle some of these complexities. "[Endocrine disrupters] provide a totally different look at a toxic substance, and that's what's so fascinating and frustrating to toxicologists," said Frederick vom Saal, a developmental reproductive biologist at the University of Missouri in Columbia. Vom Saal, an authority on the prenatal effects of hormones, is on the National Academy of Sciences committee charged with recommending research, monitoring, and testing priorities for endocrine disrupters.

Estrogen: A versatile hormone

Most known endocrine disrupters mimic or block the effects of estrogen, a hormone generated by the ovaries, testes, and adrenals that plays many roles in the body. Estrogen prompts a diverse range of effects, from ovulation and blood clotting to bone growth. It also modulates the immune system. In the fetus, estrogen plays a major role in organ development. During the early months of gestation, the balance of estrogen and other sex hormones determines whether the fetus develops male or female sex organs.

Estrogen achieves these effects by binding to receptors lodged in the nuclei of cells. Such binding turns on certain genes, which can act on the reproductive tract, skeleton, brain, liver, kidney, immune system, and the lining of the blood vessels. Remarkably, studies show that two different estrogen compounds can bind to the same receptor, yet turn on a whole different gamut of genes.

Compounds can also exert estrogen effects by binding to different estrogen receptors that stud the surfaces of some cells. Such binding has been linked to changes in the electrical activity of brain cells and the secretion of a hormone that stimulates breast milk production.

There is mounting evidence that many synthetic compounds can trigger or block estrogen effects in the body by binding to estrogen receptors. One of the first clues that synthetic compounds might exert hormone-like effects was the discovery in 1949 that crop dusters handling dichlorodiphenyltrichloroethane (DDT) had reduced sperm counts. Biologists also noticed that in regions heavily contami-

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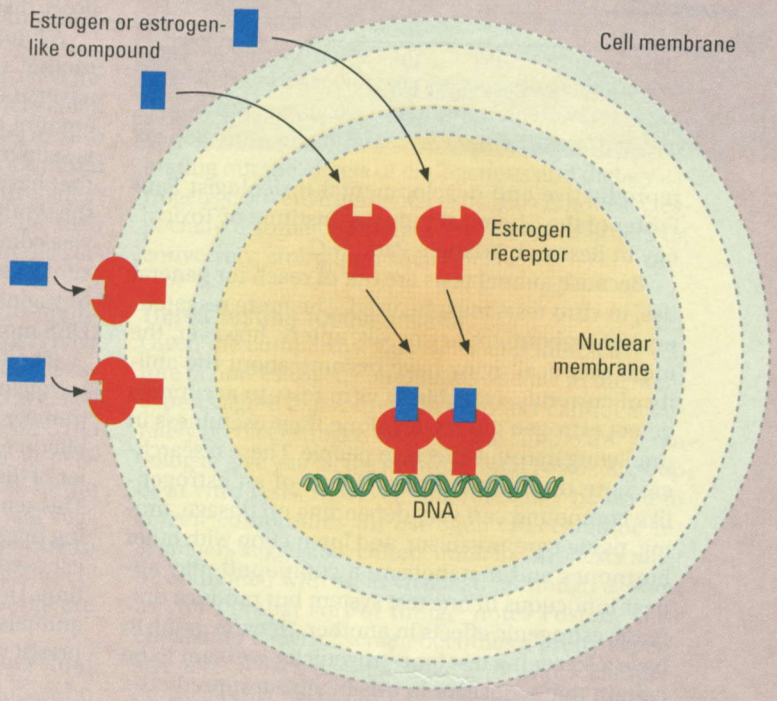
nated by DDT, gulls had deformed sexual organs. In the 1980s, when University of California–Davis toxicologist D. Michael Fry injected gull eggs with DDT, the hatchlings had the same sexual deformities as those seen in DDT-contaminated areas. These effects could also be generated by injecting the eggs with a natural form of estrogen called estradiol. Shortly after that, feminized alligators were found in a Florida lake thought to be contaminated with a DDT-like compound called dicofol. Estrogen-like contaminants in the environment have also been linked to reproductive deformities or impaired fertility in fish, turtles, and mammals (*1*).

Spurred on by the wildlife findings, lab scientists have identified more than 50 compounds that various tests suggest are endocrine disrupters. Among them are compounds found in the plastics used to package many foods and beverages; the commonly used pesticides endosulfan and methoxychlor; and several industrial chemicals, including dioxin and some polychlorinated biphenyls (PCBs) (*2*).

Surprisingly, researchers have yet to uncover a common structural feature that links this diverse group of likely endocrine disrupters. Without a telltale trait, researchers must conduct test tube or animal studies to detect hormone-like effects. These tests identify compounds likely to pose a health risk to humans. But studies showing a strong link between exposure to these compounds and various health problems in people are needed to fully assess the health hazards these compounds might induce.

How estrogens exert their influence

Estrogen and estrogen mimics trigger many of their diverse effects by binding to estrogen receptors floating in the nuclei of cells. Estrogen-bound receptors then link up with the cell's DNA to turn on certain genes, which commandeer estrogen's effects in target organs, including those of the reproductive tract, brain, liver, kidney, and immune system. Compounds also exert estrogen effects by binding to estrogen receptors that stud the surfaces of some cells. Such binding does not appear to turn on genes, but can initiate other changes in the body.



Shortcomings of current tests

Most laboratory tests for endocrine disrupters are designed to detect estrogen effects. The gold standard for animal, or in vivo, tests is to give a chemical to rodents with immature or removed ovaries (and thus without their own internal source of estrogen) over several days. The uteri of the autopsied animals are then compared with those of controls to determine whether the chemical has prompted uterine growth.

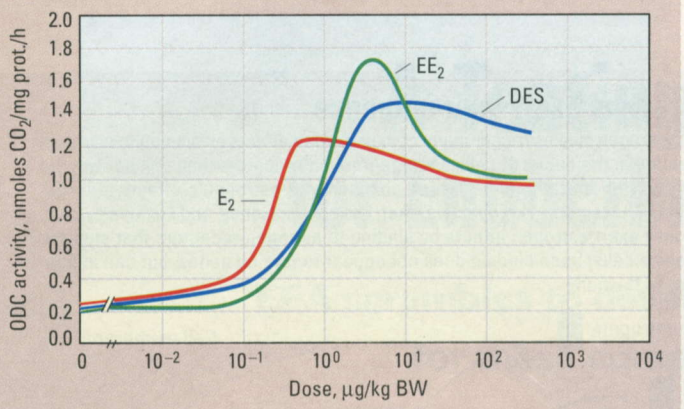
An easier and less expensive screen for estrogen activity, known as the E screen, is a test tube, or in vitro, method that measures how much a compound prompts cultured breast cancer cells to multiply. The cancer cells, which harbor estrogen receptors, grow only if estrogen or estrogen mimics are added. The amount of cell proliferation a test chemical induces over a six-day period, compared with that prompted by estradiol in the same test system, determines the compound's estrogenic rating.

Other commonly used tests for estrogen effects measure the amount of gene activity triggered by chemicals latching on to estrogen receptors in the cell nucleus. These quick assays, which usually use cultures of genetically engineered yeast or mammal cells, generally take less than a day.

But none of these tests can detect the effects of a compound on the developing fetus. The gold standard for assessing this is to administer a compound to pregnant rodents and then look for any adverse effects on the animals' progeny. Lasting up to 18 months, this test is too expensive and time-consuming to be practical for testing a large number of chemicals. If it were used to test the thousands of chemicals suspected of being estrogenic, "there would be enough work to keep labs going for the next three to four hundred years," according to

Unusual dose–response curves for estrogenic compounds

Unlike most substances in which response is proportional to dose, researchers often find that estrogen and estrogen mimics produce their greatest effects at lower doses. These dose–response curves for adult rats show an inverted U-shaped curve in the production of a uterine enzyme (ODC) in response to administration of natural estrogen (E_2) and synthetic estrogens (EE_2, DES).



Source: Reference 10.

reproductive and developmental toxicologist Paul Foster of the Chemical Industry Institute of Toxicology in Research Triangle Park, N.C.

Because animal tests are out of reach for general use, in vitro tests must be used. The more researchers learn about endocrine disrupters, however, the more skeptical many have become about the ability of currently available in vitro tests to accurately detect estrogen effects, let alone their usefulness in predicting harmful effects in people. These researchers note that, because the effects of an estrogen-like compound can vary depending on dosage, timing, tissue type, organism, and interaction with other hormones and metabolism, a compound may appear innocuous in one test system but produce dramatic estrogenic effects in another. "If we're going to have a screening test for estrogenicity, we want to be certain that a positive in this in vitro test predicts a positive in vivo response, and a negative predicts a negative," said Foster. "But now, all we can say is a positive is a maybe and a negative is a maybe. That's useless."

A compound that binds to estrogen receptors on the surface of cells, for example, will be missed in tests that merely measure the activity of genes stimulated by compounds that bind to cell nucleus receptors (*3*). A specific gene activity assay will also miss effects elicited by a compound whose estrogen binding stimulates the activity of other genes (*4*). There is also concern that what acts as an estrogen in yeast cells used for gene activity assays will not be active in mammals. A compound can also test negative in an in vitro test and yet exert powerful estrogenic effects in an animal, because the compound's breakdown product—and not the compound itself—is the estrogenic culprit.

The ability of an assay or animal test to detect estrogenic effects also varies, depending on which tissues are targeted and when and how much of the compound is administered. Some compounds block the effects of estrogen in some tissues and trigger estrogen effects in others. For example, the drug tamoxifen blocks estradiol's stimulation of breast cancer cells but promotes uterine cell growth.

Greatest effects at lowest doses

Toxicologists traditionally forecast the effects of a low dose based on extrapolations of the effects seen at higher doses. But once again, environmental estrogens stymie such efforts. Researchers often find the greatest effects are seen with the lowest doses tested. "This is a system that is programmed to work at staggering low levels of hormones," said vom Saal. "Large doses shut it off." This makes it difficult to determine the minimum dose of an estrogenic compound needed to cause adverse effects. According to vom Saal, the unusual upside-down U-shaped dose–response curve of hormones has also led many researchers to falsely label a compound an estrogen-blocker. "Even estrogen is an anti-estrogen at the doses these people are using in their studies," he said.

A fetus exposed to environmental estrogens in its mother's blood is much more prone to permanent adverse effects than an adult exposed to the same amount of the compounds. A tragic example of this is seen in the reproductive deformities and cancers that have occurred in the offspring of women given the synthetic estrogen diethylstilbestrol (DES), which was commonly used in the 1950s and 1960s to prevent miscarriages. Consequences such as infertility or vaginal cancers did not surface until daughters of DES mothers reached adulthood.

The fetus is extraordinarily sensitive to external estrogens. When vom Saal fed pregnant mice an extremely low dose of DDT or DES (in the parts-per-billion or parts-per-trillion range), the sexual behavior of the male offspring was significantly affected (*5*). This sensitivity is particularly important because during pregnancy and lactation, the developing child is exposed to a dose accumulated by the mother over time. Unlike natural estrogens produced by plants and animals, many synthetic estrogens accumulate and persist in fat tissue—sometimes for decades. Such ac-

Separating synthetic estrogens

Current tests for estrogen mimics are unable to separate the effects of synthetic estrogens from those found in food or generated by the body. But Ana Soto of Tufts University in Boston has developed a method for sifting out some types of synthetic estrogens from ovarian-generated estrogens and plant estrogens in human blood. The combined effects of these synthetic estrogens can then be tested in an E screen. The separation technique can be used to tie people's exposure to various environmental estrogens to adverse effects without the confounding interference of dietary and naturally generated estrogens present in the blood (*2*).

Soto's method uses several solvents and centrifugation to isolate estrogen compounds in blood. Liquid chromatography is then used to further separate synthetic estrogens from endogenous or plant estrogens. A pilot study suggested the method is feasible, and Soto is currently refining and validating it with other screening techniques. —M. P.

cumulations, along with other nutrients, are released into circulation and passed on to the developing embryo and newborn during pregnancy and lactation. Most animal or in vitro tests cannot predict the consequences of such stockpiles of environmental estrogens, and they do not factor into the binding of synthetic estrogens to proteins found in the blood. Such binding limits the amount of the compound that can latch onto estrogen receptors.

New complications found

The versatility of environmental estrogens also poses problems for those trying to assess their effects. Many of these compounds can bind to more than one type of steroid receptor. For example, DDT and chlordecone can bind to both estrogen and progesterone receptors. Other compounds bind to both estrogen and androgen receptors (*5*). "Some of the confusion about what these chemicals are doing is because they are actually operating through more than one hormone receptor," said vom Saal.

Two recent studies have further complicated the issue of testing for estrogen effects. John McLachlan and his colleagues at Tulane University, using in vitro tests, found that several estrogenic compounds mixed together had greater effects than the additive effect of each chemical alone. Some mixtures were 1600 times more potent than the additive effect of the single chemicals (*6*).

The applicability of McLachlan's tests to mammals has been questioned. But other researchers have also shown less dramatic synergistic effects of estrogenic compounds in turtles and breast cancer cell cultures. These results suggest that to truly assess the effects of the many environmental estrogens people are exposed to simultaneously, mixtures of the compounds may have to be tested. Vom Saal suggested regulators might want to link monitoring to testing so that they can assess the synergistic effects of the combinations of chemicals people are most likely exposed to simultaneously.

The second new complicating result comes from Swedish researchers at the Karolinska Institute, who have found a new nuclear estrogen receptor harbored by the ovary and prostate (*7*). The researchers cloned the receptor from rat tissue, and their tests suggest that some estrogens may be more inclined to bind to the novel receptor than to the classic estrogen receptor and vice versa. This is an important finding, because it suggests that "just measuring responses mediated by the classic estrogen receptor may not tell you everything you need to know," according to reproductive and developmental toxicologist Daniel Sheehan of the Food and Drug Administration's Center for Toxicological Research in Jefferson, Ark.

Lurking behind every in vitro or highly controlled animal test is the nagging doubt that it will not truly predict adverse effects in people. Such doubt is particularly prevalent in testing for the effects of environmental estrogens because—unlike test animals or tissue cultures—people are exposed to a large number of environmental hormones, including those found in their diet. Many plants, including soybeans, several seeds and grains, and certain fruits and vegetables, contain compounds that block or mimic the effects of estrogens (*8*). People also generate their own estrogens at concentrations that, in women, vary widely from one time in the month to another. Separating out the effects of endogenous and dietary estrogens from environmental estrogens is not easy, especially if some counteract the effects of others or, conversely, strengthen such effects.

Tiered testing recommended

Given the complexity, variability, and interactive nature of how estrogen compounds operate in the body, experts agree that a single in vitro test cannot accurately predict estrogen effects. "One simple test is not going to give you all the answers," said Foster. A group of in vitro tests, however, might reliably be used to screen compounds for estrogen effects, some researchers suggested. Others think in vitro tests must be combined with an in vivo test, "although as soon as you say that sort of thing," notes Foster, "your cheap and cheerful screen becomes a pretty expensive test. What's cost-effective and what's comprehensive don't necessarily fit together."

Sheehan suggested a tiered testing approach in which researchers initially test compounds with a few rapid and inexpensive in vitro tests. Chemicals that test positive in these should then be tested in animal studies. That way, "you only work your way up the level of complexity and expense with chemicals that seem, in the first few tiers, to be the worst ones," he said.

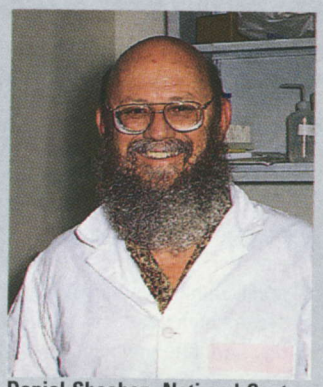
But the best set of screening tests for endocrine disrupters has yet to be identified. EPA has been bringing together stakeholders and experts from academia, industry, and government in a series of meetings aimed at developing an effective screening program for endocrine disrupters. At a meeting held in Durham, N.C., in July, the strengths and weaknesses of about two dozen current assays for endocrine disrupters were discussed, but there was no agreement about which tests to include in a screening program.

In a meeting on endocrine disruption screening and testing held in May, EPA's Lynn Goldman said the agency has recently begun requiring the testing of new chemicals for potential endocrine disruption ac-

Screening for other estrogen receptors

Chemicals that disrupt the endocrine system by binding to estrogen receptors on the cell surface instead of receptors in the cell nucleus are missed by current tests. Cheryl Watson and her colleagues at the University of Texas Medical Branch in Galveston have developed a system for detecting estrogen effects triggered by binding to cell membrane rather than nuclear estrogen receptors. The system measures the rapid secretion of the lactation hormone prolactin from cultured pituitary tumor cells. Such rapid secretion is thought to be triggered by compounds binding to estrogen receptors harbored on the surface of pituitary cells. Watson is currently developing her system into a convenient assay (*3*). —M. P.

Testing for fetal effects



Daniel Sheehan, National Center for Toxicological Research

There is currently no short-term and inexpensive animal test for the effects of environmental estrogens on the developing fetus. Daniel Sheehan of the U.S. Food and Drug Administration's Center for Toxicological Research has developed an inexpensive and short-term animal test that shows promise for quantifying the effects of an environmental estrogen compound during prenatal development. The test measures what level of environmental estrogens administered to pregnant mice prompt uterine cells in their female fetuses to multiply. Sheehan said researchers can use the test to assess whether an environmental estrogen given to the mother passes through to the fetus in a form likely to cause adverse effects (*9*).

Sheehan's assay can also be used to assess the effects of environmental estrogens stored in fat tissue and released into circulation during pregnancy by administering the estrogen compounds before pregnancy. To determine the effects of environmental estrogens released into breast milk, Sheehan added, researchers can measure the amount of uterine proliferation seen in unexposed mouse pups who are nursed by mothers prenatally exposed to environmental estrogens.

"The assay we developed is pegged to the historically accepted 'gold standard' for estrogen activity." Sheehan said. "People have confidence in that assay and know what the results mean when they see them." The assay is much more cost-effective than standard multigeneration studies, he added.

Sheehan has used his animal model to construct dose–response curves for a dozen estrogenic chemicals given at various developmental stages of the mouse. —M.P.

tivity using the gold standard in vivo assay, which measures uterine cell proliferation in rodents. As alternative test methods are developed and validated, however, testing requirements may change.

Legislation sets testing timetable

Driving EPA actions are two new acts of Congress. The Food Quality Protection Act of 1996, enacted in July, requires EPA to test all pesticide chemicals, including the inert ingredients of the pesticides, for endocrine disruption effects. The act also specifies that EPA can require the testing of "any other substance that may have an effect that is cumulative to an effect of a pesticide chemical" if EPA determines a substantial population may be exposed to such a substance. According to Goldman, EPA is interpreting the "cumulative effects" clause to mean testing of chemicals found in association with pesticides or with the same effects.

The Safe Drinking Water Act Amendments of 1996, which were signed into law in August, authorize EPA to "develop and implement a program to identify and regulate substances that may have effects on humans similar to those produced by naturally occurring estrogen or other endocrine effects." Neither of these acts of Congress gives EPA any new authority to test for endocrine disrupters. The agency already had this authority under the Toxic Substances Control Act and the Federal Insecticide, Fungicide, and Rodenticide Act. But both laws give EPA highly specific time limits: They require the agency to develop a screening and testing strategy for endocrine disrupters within two years and implement the strategy within three years.

Will EPA be able to meet this congressional mandate by the close of 1998? Goldman thinks it is a feasible goal, although others, such as Robert Kavlock of EPA's National Health and Environmental Effects Research Laboratory, are more skeptical. He noted that EPA has missed other congressional deadlines set in the past.

Most researchers in the field will not even hazard a guess as to when an effective screening program for endocrine disrupters is likely to be formulated. As Foster concluded, "Screening compounds for endocrine disruption is not impossible, but we're not there yet." Even Goldman admits that whatever screening strategy EPA devises within two years is likely to evolve over time as more scientific findings are reported. "Science is an ongoing process, and regulatory science has to move along with it," she said. Like other scientific techniques that are praised, improved, or discarded as more research is done, endocrine disruption assays will have to undergo the ultimate screen: the test of time.

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