Studijní materiál
Ekotoxikologie
* Prostudovat (nejlépe celé), zejména (!) žlutě vyznačené pasáže

THE LEARNING CURVE

Researchers say that some chemicals have unexpected and potent effects at very low doses — but regulators aren't convinced.

BY DAN FAGIN

Near the end of an adventurous life spent wandering the fortress towns of central Europe, clashing with bloodletters and other tradition-bound healers of the day, the irascible sixteenth-century physician Paracelsus wrote a defence of his unorthodox use of mercury, opium and other potentially dangerous medicines. "All things are poison, and nothing is without poison: the dose alone makes a thing not poison," he wrote. Centuries later, after many of his once-radical ideas found wide acceptance, Paracelsus's pronouncement would be distilled into a pithy phrase that became foundational dogma for the modern science of toxicology: "the dose makes the poison."

The contemporary interpretation of Paracelsus's famous declaration, for which he is often called the father of toxicology, is that dose and effect move together in a predictably linear fashion, and that lower exposures to a hazardous compound will therefore always generate lower risks. This idea is not just a philosophical abstraction; it is the core assumption underlying the system of chemical-safety testing that arose in the mid-twentieth century. Risk assessors typically look for adverse effects of a compound over a range of high doses and, from there, extrapolate downwards to establish health standards — always assuming, like Paracelsus, that chemicals toxic at high doses are much less risky at lower, real-world levels.

But what if the Paracelsian presumption is wrong? What if, for a large and potent class of compounds, lower doses pose higher risks? A growing number of academic researchers are making just such a claim for endocrine disrupters, a large group of synthetic chemicals able to interact with cellular hormone receptors. These compounds, which range from the common weed killer atrazine and the plasticizer bisphenol A (BPA) to the antibacterial agent triclosan (used in cleansers) and the vineyard fungicide vinclozolin, don't play by the usual rules of toxicology. On the basis of conventional high-dose testing, regulators have set maximum acceptable levels for each of them that assume all doses below that level are safe. But academic researchers who have studied a wider range of doses, including very low ones found in the everyday environment, say that their experiments usually do not generate the tidy, familiar 'ski-slope' dose-response graphs of classic toxicology. Instead, most endocrine disrupters have 'non-monotonic' dose-response curves, meaning that their slopes change at least once from negative to positive, or vice versa, forming 'U' shapes, inverted 'U's or even stranger shapes that resemble undulating Chinese dragons (see 'Curious curves').

"We're seeing that for every one of these compounds we test, there will be a non-monotonic response — every one!" says Frederick vom Saal, a neurobiologist at the University of Missouri-Columbia, who has been sounding the alarm about endocrine disrupters since the 1970s. "Low doses of endocrine disrupters act in ways that are totally unpredicted by the traditional approaches of toxicology." Vom Saal and his colleagues believe that very low doses of these compounds in the environment are contributing to a wide range of human health problems — including obesity, diabetes, cancer, cardiovascular disease, and infertility and other disorders related to sexual development.

Many toxicologists, however, are not convinced — especially those in industry or government who have spent their careers deeply involved in traditional risk assessment. Although they acknowledge that endocrine disrupters have unusual toxicological quirks, they say that the work of vom Saal and like-minded researchers is still insufficiently replicated, too reliant on unvalidated assays and too focused on end points such as organ weight, precancerous growths and changes in the activity of genes and proteins, which may not pose significant health threats. "If we're going to take this seriously, we need to have some evidence of a real phenomenon that happens not just in the hands of one researcher and one test, something repeatable that can stand up to scientific scrutiny about how it could lead to real health effects we want to avoid," says Lorenz Rhomberg, a toxicologist at the environmental-consulting firm Gradient in Cambridge, Massachusetts. Rhomberg also serves as a consultant on endocrine disrupters to the American Chemistry Council, an association of chemical manufacturers.

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Vom Saal and his colleagues counter that this is precisely the type of systematic evidence they can now provide, thanks to a boom in endocrine-disrupter research. The most comprehensive review yet of the field[1], published in March, included more than 600 studies — almost half of them published within the past five years — and found credible evidence of non-monotonic responses with low-dose health effects in 18 endocrine disrupters, including BPA, atrazine and vinclozolin. "We kept hearing from our critics that there aren't enough examples to prove this phenomenon is real, so we took that as a challenge," says Laura Vandenberg, a postdoctoral fellow at Tufts University in Medford, Massachusetts, and the lead author of the review.

Government officials in Europe and the United States are paying attention. "I find the Vandenberg review to be quite compelling and quite convincing," says Linda Birnbaum, director of the US National Institute of Environmental Health Sciences (NIEHS) in Research Triangle Park, North Carolina. In an April editorial in the NIEHS-published journal *Environmental Health Perspectives*, Birnbaum argued that "it is time to start the conversation" about incorporating low doses and non-monotonic relationships into regulatory decisions[2]. At a European Commission scientific conference on endocrine disrupters this June in Brussels, delegates failed to reach a consensus on the importance of non-monotonicity at low doses but they did agree that existing regulations need to be stricter, according to Björn Hansen, who heads the chemicals unit at the commission's Directorate-General for Environment in Brussels. In the United States, meanwhile, the Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA) are showing a new willingness to at least discuss the issue — although they say that major regulatory revisions are not on the table for now.

Big changes are unlikely, some observers suggest, as long as the field remains so polarized. "There is a very large divide out there between the risk assessors and the endocrine scientists," says Thomas Neltner, who studies chemical food additives at the non-profit Pew Health Group in Washington DC, which has been trying to arrange a rapprochement through a series of scientific meetings. "Our feeling is that the two sides have been talking past each other."

EARLY SIGNS

For as long as there has been controversy over the effects of endocrine disrupters, vom Saal has been at the centre of it. Lean and intense, the 67-year-old native New Yorker is an amateur pilot who flies his Cessna to scientific conferences and is not shy about tangling with his critics wherever he lands. As a postdoctoral fellow at the University of Texas at Austin in the 1970s, vom Saal was startled to discover that subtle variations in sex-hormone levels in the womb could have life-long effects on mice. A female mouse positioned between two males in the uterus would later, as an adult, display significantly more 'masculine' characteristics, such as aggression, than would a female surrounded prenatally by other females, vom Saal found[3]. The apparent cause: a minuscule amount of extra testosterone released by the neighbouring male fetuses.

Experimenting first with natural hormones and the synthetic oestrogen diethylstilbestrol (DES), vom Saal found that male mice exposed prenatally to very low levels of DES developed heavier prostates than unexposed mice — making them more vulnerable later in life to prostate disease, including cancer. Strangely, however, he found that higher doses of DES did not trigger the same effect[4]. It was one of the first non-monotonic dose-response curves mapped for an endocrine disrupter. Since then, vom Saal and his Missouri colleague Wade Welshons have identified similar non-monotonic responses from a variety of endocrine disrupters, most notably BPA[5], a ubiquitous ingredient of polycarbonate plastics and epoxy coatings, including in food packaging.

Vom Saal's early work helped to generate a torrent of international interest in BPA, including an ultimately successful campaign by activists in the United States, Canada and some European countries to halt its use in baby bottles and toddlers' sippy cups. It also helped to inspire a legion of researchers to look for — and often find — other endocrine-related effects in animals exposed to very low levels of BPA and other hormone mimics. At Tufts, for example, cell biologist Ana Soto won notoriety for discovering that early exposure to BPA can alter the development of mammary glands in mice and rats, spurring the growth of oestrogen receptors and leading to precancerous growths and non-invasive cancers[6]. In Spain, another cell biologist, Angel Nadal of Miguel Hernández University in Elche, exposed human

pancreatic cells to BPA and mapped non-monotonic relationships between dose levels and altered glucose metabolism, a key risk factor for diabetes and obesity[7]. Epidemiologists jumped into the fray, too. They found associations between urine BPA levels and obesity in children[8], and linked other endocrine disrupters to incidence of diabetes[9].

Their studies, and many others, depict a weird world of endocrine disruption that is as different from traditional toxicology as quantum mechanics is from the staid clockwork of Newtonian physics. When even minuscule quantities of BPA and other disrupters interact with hormone receptors at crucial moments in development — activating, jamming, hijacking or otherwise messing with their normal function — they can give rise to strange-looking experimental results, especially when other hormones are thrown into the mix.

At the University of Illinois at Chicago, for example, reproductive physiologist Gail Prins implants prostate-like glands, grown from human stem cells and mixed with mouse tissue, into young mice, and then feeds some of them very low doses of BPA. As the mice age, Prins also gives them low doses of oestradiol, a naturally occurring hormone that becomes more potent in human males as they age and is a known risk factor for prostate cancer. Between 35% and 40% of the mice fed BPA plus oestradiol have developed prostate cancer, compared with 10% of the mice that were given oestradiol only. Her working theory is that BPA binds with oestrogen receptors in prostate stem cells, reprogramming genes in ways that leave the cells more sensitive to oestradiol later on. "What's remarkable," she says, "is that we're working with very low doses that are definitely within the range of normal human exposure." She plans to publish her results in early 2013, as soon as she has collected more data.

The interplay of these types of receptor mechanisms can generate bizarre dose-response relationships, many of which are still being mapped out. Earlier this month, vom Saal's group at Missouri published the first full non-monotonic dose-response curve for the widely used plastics ingredient known as DEHP, or di(2-ethylhexyl) phthalate[10]. The Missouri team subjected 78 pregnant mice to an extremely wide range of DEHP doses — from 0.5 micrograms per kilogram of body weight per day all the way up to 500,000 micrograms. They found that the animals' testosterone levels rose or fell in surprising ways, altering sexual development depending on the dose received. For male offspring, for example, the dose-response curve looked something like the profile of a craggy mountain. Serum levels of testosterone rose between the 0 and 0.5 microgram dose levels, then fell slightly at 1, rose again through 5 and 500 before declining again at 50,000 and plunging at the 500,000-microgram dose level. The highest dose, in fact, was virtually identical to the results for unexposed controls. Seeking to deflect another potential line of attack from his critics, vom Saal conducted a 'goodness of fit' statistical analysis confirming that a non-monotonic curve best fits his data.

The biochemical mechanism behind the strange DEHP curve is unknown — vom Saal says that it awaits further study — but researchers have worked out the specific causes of other non-monotonic curves. One of the best understood examples involves not a pollutant, but a drug: the chemotherapy agent tamoxifen, which binds to oestrogen receptors in breast cells and has a dose-response curve shaped like an upside-down U. Very low doses have little effect on cancer cells, but as the drug builds up in breast tissue it actually stimulates tumour growth, triggering a painful 'flare' period for patients. When tamoxifen levels grow high enough to occupy most of the available oestrogen receptors, the effect reverses and the drug begins to inhibit the cancer cells' growth. "All of this is very well known to endocrinologists," says Thomas Zoeller of the University of Massachusetts Amherst, who studies the effects of endocrine disrupters on the thyroid. "Non-monotonic dose-response is a fact of life."

A turning point was a 2009 scientific statement by the Endocrine Society in Chevy Chase, Maryland, the first in its 95-year history. It called endocrine disrupters a "significant concern to public health"; endorsed stricter regulations; acknowledged non-monotonic responses; and declared that "even infinitesimally low levels of exposure — indeed, any level of exposure at all — may cause endocrine or reproductive abnormalities"[11]. Seven other scientific bodies then joined the Endocrine Society in a letter of concern published last year in *Science*[12]. "That's as mainstream as you can get. It really changed the nature of the game," says another long-time researcher in the field, molecular biologist Bruce Blumberg of the University of California, Irvine.

PUT TO THE TEST

Critics, however, say that the mere existence of non-monotonic responses and low-dose effects is not the point. What matters is the extent of the health concerns they raise. "There is non-monotonicity, but the question is, is it toxicologically relevant?" asks Jason Aungst, a supervisory toxicologist with the FDA's Center for Food Safety and Applied Nutrition in Silver Spring, Maryland. He and a senior toxicologist at the EPA, Earl Gray, argue that the low-dose health effects identified in studies by vom Saal, Soto and others are still relatively rare and have not been conclusively linked to major health problems. Low-dose effects that are more clearly harmful, such as organ deformities, are usually monotonic, and can be identified under current regulatory testing protocols, according to Gray. "You could never say that non-monotonicity doesn't happen, but as far as its relevance to risk assessment, we really haven't seen it in the high-quality studies," he says.

> "LOW DOSES OF ENDOCRINE DISRUPTERS ACT IN WAYS THAT ARE TOTALLY UNPREDICTED BY THE TRADITIONAL APPROACHES OF TOXICOLOGY."

The schism in the field is in part a result of the different types of tests that academic researchers and risk assessors carry out. Many of the private testing labs hired by manufacturers seeking regulatory approval for new products are not equipped to do the radioimmunoassay analyses required to measure extremely low chemical concentrations. Nor do private labs typically look for the complex biochemical changes, such as alterations in protein levels, that are now part of the standard tool kit of researchers such as Zoeller, Soto and vom Saal. Instead, agency-mandated guideline tests are standardized; involve simpler assays that are easier to replicate; use higher numbers of test animals; and generally seek to identify more obvious health problems, such as acute toxicity, cancer and physical deformities. "We do validated studies, the basic researchers don't," says Rochelle Tyl, a developmental toxicologist at RTI International, a firm in Research Triangle Park that conducts research for clients such as regulatory agencies and chemical manufacturers. "That doesn't mean they're wrong, it just means they're doing work that hasn't been validated."

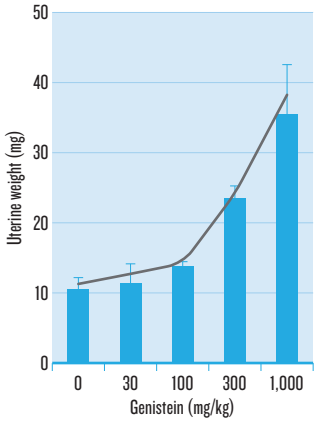
Yet even when government and industry-hired scientists look for low-dose effects, they often don't find them. For example, Tyl[13] (who conducted her studies for two industry groups) and Gray[14] have each tested BPA at very low doses without finding the potent developmental effects identified by vom Saal, Prins and others. Vom Saal and his allies counter that the Tyl and Gray studies were insensitive to low-dose effects because their positive-control animals, which were

CURIOUS CURVES

Researchers have found that many endocrine-disrupting chemicals do not generate the standard monotonic dose-response curves seen for other types of compound.

MONOTONIC CURVE

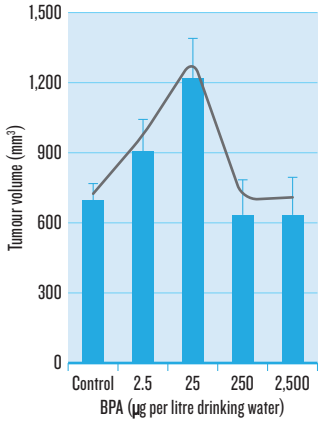
In some cases, dose and response increase together. The plant oestrogen genistein, for instance, causes the mouse uterus to increase in weight.



SOURCE: Ohto, R. *et al. J. Toxicol. Sci.* **37,** 879–889 (2012)

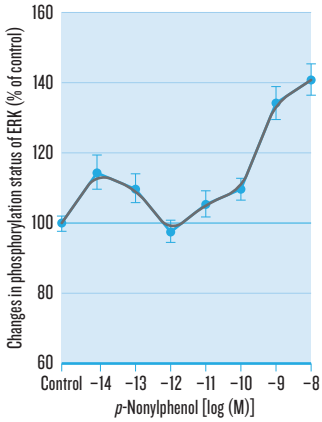
NON-MONOTONIC CURVES

Mice exposed to moderate doses of bisphenol A develop the largest tumours. Moderate and high doses are thought to induce tumour-cell proliferation, but high doses also trigger cell death.



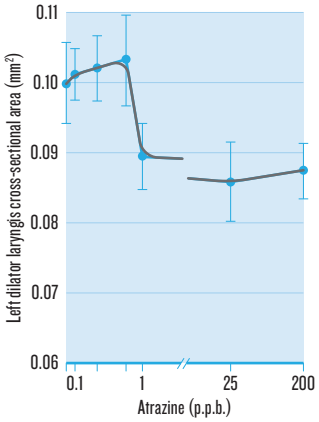
SOURCE: Jenkins, S. *et al. Environ. Health Perspect.* **119,** 1604–1609 (2011)

The oestrogen mimic *p*-nonylphenol stimulates the ERK cell-signalling pathway at low and high doses. Interactions with hormone receptors and other membrane proteins explain the complex shape of the curve.



SOURCE: Bulayeva, N. N. & Watson, C. S. *Environ. Health Perspect.* **112,** 1481–1487 (2004)

Above a certain dose, the herbicide atrazine causes the larynx muscle to shrink in male frogs. But the effect does not increase at higher doses.



SOURCE: Hayes, T. A. *et al. Proc. Natl Acad. Sci. USA* **99,** 5476–5480 (2002).

given oestradiol alone, received doses that were much too high. The dispute has been harsh and public, playing out at conferences and in a seemingly endless series of sharply worded journal articles, rebuttals, counter-rebuttals and counter-counter-rebuttals. The arguments became so heated that Tyl stopped doing BPA work. "I gave up BPA when it ceased to be scientific and became personal," she says. "It became flag-waving and it became political."

Largely because of the Tyl and Gray studies, neither the FDA nor the EPA have altered their risk assessments for BPA. The FDA still says that BPA has no adverse effects at levels below 50 milligrams per kilogram of body weight per day — a level that vom Saal contends should actually be two million times lower, at 25 nanograms. Both agencies, however, are now cooperating on a much larger study designed to settle the dispute. The newly launched US$20-million study, led by the NIEHS and the FDA's National Center for Toxicological Research, is the most ambitious effort ever to look for non-monotonic dose-response curves that include very low doses. Last month, researchers began hand-feeding BPA to about a thousand rats at five dose levels ranging from 2.5 micrograms per kilogram of body weight up to 25,000 micrograms, plus two positive-control groups (which received much lower oestradiol doses than either Tyl or Gray used) and an unexposed control group. Vom Saal, Zoeller and other academics will be participating in the tissue analysis, allowing them to look for an array of health effects, such as metabolic changes in the prostate and mammary glands, that go well beyond those in standard regulatory protocols.

But with the results of the BPA mega-study not expected for at least five years, a major rewriting of chemical regulations to take non-monotonic low-dose effects into account still seems distant. The European Commission has to meet a self-imposed deadline of December 2013 to draft the first governmental criteria defining endocrine disruption, but without a scientific consensus on the issue the criteria may end up addressing only high-dose effects, predicts Andreas Kortenkamp, a toxicologist at Brunel University in Uxbridge, UK, who has been advising the European Commission. In the United States, meanwhile, the EPA and FDA have convened a joint working group to review the evidence accumulating in the peer-reviewed literature, but "not only is the jury still out, the jury hasn't even had a chance to look at the evidence yet", says Rita Schoeny, a senior science adviser at the EPA in Washington DC.

Some veterans of the field have decided not to wait, and are collaborating on what amounts to an effort to bypass the regulatory system. They have written a paper[15], scheduled to be published in January in the journal *Green Chemistry*, that gives industrial chemists detailed advice on how to screen newly synthesized compounds for endocrine-related effects, how to test at very low doses and how to look for non-monotonic dose-response curves. The paper, and a companion website, are aimed at trying to head off potentially harmful endocrine disrupters before they reach the marketplace.

An ancillary benefit, vom Saal hopes, will be to increase the pressure on regulatory agencies to curb exposures to compounds already on the market. "We're saying that if you care about developing a safe chemical, here's what you should do," says vom Saal. "Who could argue with that?" ■

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