Test Tube Toxicology

New tests may reduce the need for animals in product safety testing

By RICK WEISS

Wanting nothing more than to darken her eyelashes, "Mrs. Brown" went blind in the spring of 1933. She suffered constant pain for three months until her corneas sloughed off, all for using an eyelash dye that promised to "radiate personality." Her experience earned her a place in a Chamber of Horrors exhibit presented to members of Congress by the then-fledgling Food and Drug Administration (FDA) as part of a successful campaign to pass the Food, Drug and Cosmetic Act of 1938.

Countless such tragedies have probably been averted since that act gave the FDA the authority to prohibit the sale of harmful cosmetics. But the legacy of "Mrs. Brown" (as she is referred to in FDA archives) remains a painful one for the more than 100,000 rabbits each year that are, under the act's provisions, subjected to similar fates.

Here is what happens: Rabbits are removed from their cages and held firmly while their eyelids are pulled back and a measured dose of a suspected eye irritant is squirted onto the eye. The rabbits' eyes are then observed after 24, 48 and 72 hours for redness, blistering, bleeding or blindness.

In recent years, however, there has emerged a movement aimed at replacing such animal tests with *in vitro*, or test tube, alternatives. While federal regulatory agencies still require animal tests as the definitive benchmark of human toxicity, several *in vitro* alternatives are now being considered as potential substitutes for some of these tests.

Replacing animal tests such as the classic Draize test for ocular irritancy, described above, "used to be considered a flaky, humane idea," says Henry Spira, a leading spokesman for the movement against animal testing. "But today, *in vitro* has moved into the mainstream."

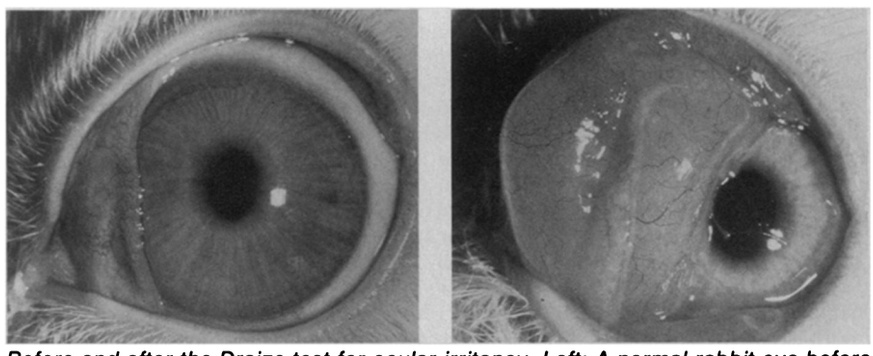
As evidence of that ever-widening mainstream, Spira need do no more than note the existence of the Johns Hopkins Center for Alternatives to Animal Testing (CAAT), in Baltimore, where he was interviewed during a recent scientific symposium. The university-based research institute is solely devoted to the study of new methods for testing the safety of household chemical, cosmetic and therapeutic products.

The symposium attracted more than 200 university and industry researchers, government regulators and private citizens to provide an update on the status of *in vitro* skin and eye toxicity testing. It was highlighted by the unveiling of a newly developed line of "Living Skin Equivalents" — mass-produced globs of living, growing human skin that have no qualms about being used for toxicity testing.

According to symposium participants, more than 100 *in vitro* toxicology tests, most of them using cell or tissue cultures, are under development — evidence that many scientists, including leading researchers in some of the largest cosmetic companies, are now in agreement on the benefits of *in vitro* toxicology tests.

However, some researchers and activists say, federal regulatory agencies are failing to provide the leadership and incentives needed to nurture that toxicological transition. Critics say that while the agencies espouse their support for *in vitro* alternatives, the important work of developing new standards is entangled in a bureaucratic jungle of task forces and study groups.

For example, the Environmental Protection Agency (EPA) has a committee looking into *in vitro* validation, the FDA has another, and the National Institutes of



Before and after the Draize test for ocular irritancy. Left: A normal rabbit eye before exposure to a suspected irritant. Right: A severe inflammatory reaction in a rabbit eye after laboratory exposure to a chemical irritant. Damage can include blood vessel breakdown, corneal bulging and opacity.

Health (NIH) sponsors one as well. In addition, there exists an Interagency Animal Research Committee with representatives from the NIH, the FDA, the EPA, the Department of Energy and the Department of Defense. But while some of these committees have existed for years, they haven't even come close to agreeing on a standardized list of acceptable *in vitro* alternatives. Moreover, the changes that *have* been suggested or initiated vary significantly from agency to agency.

"Hope springs eternal that something will happen," says Spira, the animal rights organizer. "But people get bored with committees after a while."

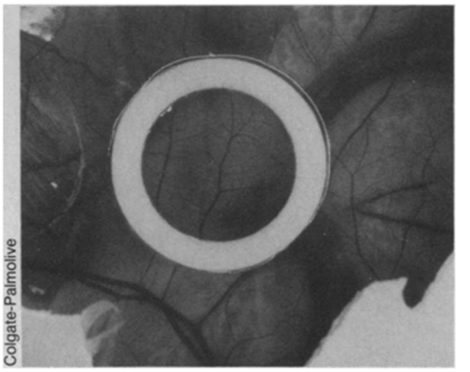
Spira knows how to break up such boredom. In one of the turning points of the animal rights movement, he organized a 1980 protest against Revlon, Inc., the cosmetics industry "flagship." He inspired individuals from more than 400 groups to dress in rabbit costumes and march outside Revlon's corporate offices in opposition to the company's use of the Draize test. He ran full-page newspaper ads depicting bandaged white rabbits asking, "How many rabbits does Revlon blind for beauty's sake?"

Six months later Revlon initiated a research program at Rockefeller University in New York City to look for alternatives to the Draize test. Within months, other cosmetic companies contributed hundreds of thousands of dollars to similar programs, and the search for *in vitro* alternatives got seriously under way.

Today the search for appropriate *in vitro* alternatives is almost in vogue. As part of its official, widely publicized testing policy, for example, Proctor and Gamble is "committed to pursue actively the development, validation and adoption of new testing methods that serve to eliminate the need for the use of animals, to reduce the number of animals used, or reduce the distress to which animals are subjected." Similarly, Avon's stated objective is "to eliminate animal tests for product safety."

Such commitments are more than token gestures, many activists agree. If nothing else, they say, companies are finding that *in vitro* tests can have a number of advantages over traditional animal tests. According to the Congressional Office of Technology Assessment, for example, *in vitro* tests are approximately one-tenth the cost of animal tests — averaging $50,000 per product as opposed to $500,000 when animals are used.

Moreover, *in vitro* tests can be more precise than many animal tests. John Farber, a professor of pathology at Jefferson Medical College in Philadelphia, has been using *in vitro* liver cell cultures to test the toxicity of the common analgesic, acetaminophen. "We, as well as many other groups, have been [able] to manipulate the cultured hepatocytes in ways that are quite impossible with an intact animal to probe the mechanisms of irreversible injury by hepatotoxins," he says.



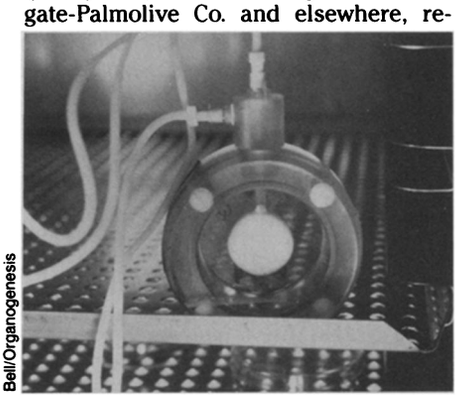
Colgate-Palmolive

The CAM test: A few drops of a suspected irritant are placed on an exposed membrane in a live chick egg and damage to tiny blood vessels is scored. A Teflon ring keeps the test material in one area.

A related benefit of *in vitro* testing, he notes, is the possibility of learning more about the molecular mechanisms that underlie inflammation, membrane damage and tissue toxicity. His research, for example, has led to a new biomolecular model of membrane damage. If his findings are confirmed, they may lead to new strategies for preventing or treating tissue injury.

Membrane damage is one of the early biological "markers" of tissue toxicity, and researchers seek evidence of it in a number of *in vitro* tests. The so-called Neutral Red Uptake test, for example, which is being investigated in several U.S. and European laboratories, uses a biological stain to look for evidence of membrane damage in culture-grown human skin cells. Similarly, a test being developed at the Medical College of Pennsylvania in Philadelphia exposes cultured dog kidney cells to a suspected toxin, then looks for increased permeability to a fluorescein dye due to membrane damage.

In the chorionioallantoic membrane (CAM) test, under investigation at Colgate-Palmolive Co. and elsewhere, researchers remove a small piece of eggshell from a chick egg, leaving the heavily vascularized, underlying membrane intact. They place a few drops of a suspected toxin, dissolved in saline, on the exposed membrane and measure the amount of blood-vessel breakdown as an indicator of toxicity.



Bell/Organogenesis

A Test Skin system prototype showing a mass of living skin growing in its mold in an incubator. Tubes provide nutrients.

Other *in vitro* tests are more specific for a particular type of damage. Researchers at Ohio State University in Columbus are using sensitive, enzyme-based antibody tests to detect the production of a substance called C-reactive protein in white blood cells grown in culture with liver cells. C-reactive protein is an early indicator of tissue damage and a key element in the inflammatory response. Other tests take advantage of recent progress in molecular biology, allowing researchers to measure minuscule amounts of messenger RNA (mRNA) indicative of the production of telltale proteins in damaged cells.

Powerful enzymes called proteinases, for example, are key indicators of and contributors to damage in skin. They activate and are important in blood clotting, cell movement and tissue repair. Tests that look for proteinases can be confounded, however, because many cells can also release proteinase *inhibitors* that quickly bind to these enzymes, making them difficult to detect. Rather than looking for the hidden enzymes themselves, researchers are using molecular biological techniques to spot increases in mRNA specific for those enzymes.

"With the complexities of this [protein synthesis] system, many of us who were interested in the biomedicine end have almost by necessity become molecular biologists," says Gerald Lazarus, of the University of Pennsylvania. Although it is a complicated field, he says, "We've gotten into this area because it gives us very crisp, critical and specific information, eliminating many of the problems inherent in biochemical studies."

There are disadvantages, however, to such specific measures of toxicity. Indeed, one of the fundamental problems with *in vitro* models is that they fail to mimic the complexity of the whole, living organism. Thus the premier caveat among *in vitro* toxicologists: Never settle for the results of a single test.

"Any risk assessment of human ocular irritation or any other type of toxicity is going to have to be based on a spectrum of data generated from a battery of tests," says John Frazier, a professor of environmental health at Johns Hopkins and associate director of CAAT.

For example, says Lazarus, "Skin is a complex organ made of multiple constituents. But many of its individual cellular components can be cultured and studied separately." Taken together, such results can be good predictors of human toxicity.

Other researchers, however, have

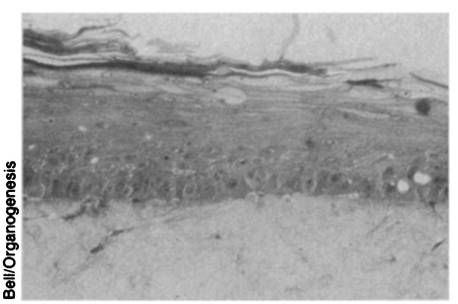
sought to go one step farther by combining the various components of skin into a complex, living laboratory specimen that can be tested as a unit. Eugene Bell, professor emeritus at the Massachusetts Institute of Technology and Chairman of Organogenesis, Inc., a biotechnology company in Cambridge, Mass., unveiled at the symposium a "Living Skin Equivalent" especially adapted for use in toxicology testing. Living Skin Equivalent, a complex, cultured human skin cell product, was originally developed by Organogenesis as an immunologically neutral skin graft for burn treatment.

"We all knew that there was a need for viable human skin to test substance toxicity, irritation, allergenicity, to test the rate of entry of substances into the body through the skin and to test the development of cosmetic and pharmaceutical agents," says Bell.

Commercially available human epithelial cultures are insufficient models for many such tests, he says, because they include only the outermost cells of what is in fact a very complex, multilayered tissue. His new product, which he calls "Test Skin," is in his words "a complex hybrid organ. It has a synthetic skeleton and circulatory system, but is covered with a living integument. It feels like a piece of skin."

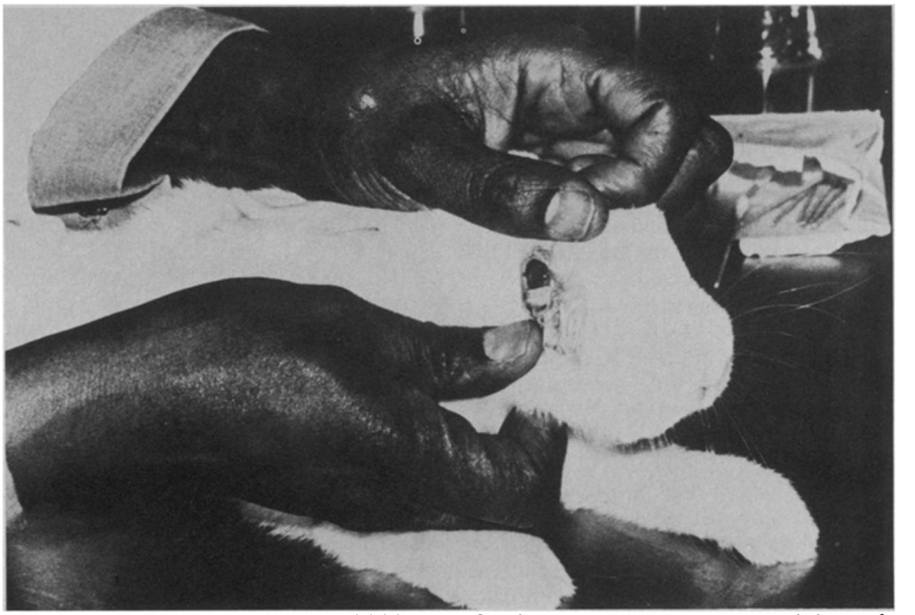
Bell makes Test Skin in a two-stage process. First, he mixes cultured dermal fibroblast cells with appropriate nutrients and other biological molecules, and pours them all into a mold. Over a period of a few days, this gel condenses into a "dermal equivalent," or a mass of cells similar to the deeper layers of skin.

Later, human keratinocytes – the type of cells that form the outer layer of skin – are cultured onto the dermal equivalent. They multiply and spontaneously organize themselves into a multilayered, differentiated epidermis within about four days. After three to four weeks, a complete basal lamina – a layer of specially arranged cells – is formed between the epidermis and dermis. The basal lamina, present in living skin but never before created *in vitro*, is believed to be an important region governing toxin penetration and the inflammatory response.



Bell/Organogenesis

Microscopic cross section of Living Skin Equivalent showing underlying dermal equivalent covered by a multilayered, maturing epidermis.



Laboratory worker examines a rabbit's eye after its exposure to a measured dose of an irritant.

"This making of skin, it's a bottle-filling operation," says Bell, adding that pigment-containing cells called melanocytes can be added just before keratinization, enabling the skin to tan after exposure to sunlight. "It even repairs itself when wounded," he says. He predicts that Test Skin will be commercially available for use in *in vitro* toxicology within the next 12 months.

Not everyone at the Baltimore symposium was sold on Bell's product. Paul Wegener of San Diego-based Clonetics, Corp., a maker of cultured epithelial cells, says there may be advantages to testing different cell types separately. "And one thing you can tell about Bell's product," he says: "It's not going to be cheap."

But the successful modeling of a tissue system even hinting of the complexity of human skin bodes well for the future of *in vitro* testing. "I think that this is just the very beginning of a method to create hybrid organisms that will be useful for testing," Bell says. "We are really primitive at this point."

If the goal is to become less primitive, and a decline in the use of animal tests is one measure of that goal, then according to Animal Rights International, a New York City-based coalition of animal rights groups, progress is being made.

The two largest independent toxicology labs in the United States, Battelle in Columbus, Ohio, and Hazelton Laboratories in Vienna, Va., are actively developing *in vitro* alternative tests. The Soap and Detergent Association in New York City has a Draize-alternative validation project, as does the Washington, D.C.-based Cosmetic, Toiletry and Fragrance Association.

Among government agencies, the National Toxicology Program of the Department of Health and Human Services is evaluating *in vitro* systems and has asked for proposals on alternative test development. And the National Institute of Environmental Health has already spent more than $70 million since 1981 toward developing a toxicological database that could serve as a standard for various *in vitro* tests. Moreover, no fewer than five scientific journals devoted to *in vitro* methods have been born in the past two years.

So far, however, regulatory agencies – the key link between basic research and commercial use – have given few clues about which, if any, *in vitro* tests they anticipate may be accepted as alternatives to current tests. And although committees abound, says Kailash Gupta, of the Health Sciences division of the Consumer Product Safety Commission, "None of the agencies as far as I know has seriously sat down and said that these are the criteria that alternative tests should meet to be accepted."

Indeed, wrote Spira in a recent letter to federal regulators, although "regulatory agencies have publicly stated their support for alternatives ... most of the regulatory agencies' actions have sent quite a different message to the industry: that, for regulatory purposes, it seems impossible even to begin replacing traditional methods with alternatives."

For example, he notes, EPA still refuses to accept a more humane version of the Draize test, the Low Level Eye Irritation (LLEI) test, declaring "more scientific evidence is needed before we will accept the change." But EPA fails to define precisely what evidence would be satisfactory, Spira says. EPA's lack of acceptance of the LLEI test is especially disturbing, he says, since it represents not a replacement but only a modification of an existing, traditionally accepted test. He notes

that the test has been studied for more than a decade, its results have been documented in peer-reviewed journals and it has been designated a standard method by the American Society for Testing and Materials, a nationally respected organization devoted to developing test standards.

Sen. William Proxmire (D-Wis.), in a letter to EPA Administrator Lee Thomas, recently expressed his dismay that "the Agency may not be doing everything it possibly can to replace traditional methods of testing with alternatives."

The EPA has also been criticized for continuing to require the so-called LD_{50} test as a measure of mammalian toxicity. The test has been described by Gerhard Zbinden, an internationally renowned toxicologist and director of the Institute for Toxicology in Zurich, Switzerland, as "a ritual mass execution of animals." Each LD_{50} test requires that 40 to 200 animals be exposed to a range of concentrations of a potential toxin in order to determine the dose at which 50 percent of the animals will die.

"Clearly, such experiences reinforce industry's perception that there is no clear regulatory procedure. . . .for replacing animal tests with appropriate alternatives," Spira says. "These ambiguities produce a counter-incentive for industry to invest significant resources to develop, validate and implement alternatives."

Theodore M. Farber, director of the EPA's toxicology branch, confirms that the LD_{50} is still required. However, he says, "We have formed a committee that will be looking for some acceptable alternatives to the LD_{50}. We are also actively looking at the Low Level Eye Irritation test." Alternatives might be identified and validated for at least limited use as early as this spring or summer, he says.

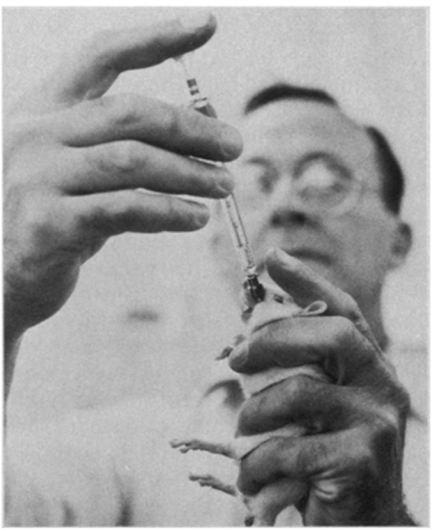
Bureaucratic sluggishness is not the only factor slowing acceptance of *in vitro* alternatives; difficult scientific hurdles remain. Uppermost among them is the need to design objective validation criteria for new test methods.

For example, to test the validity of two new *in vitro* tests that make use of different tissue cultures, each culture might be exposed to a standardized selection of common irritants – from the most innocuous to the most corrosive – and the results compared to traditional animal test results. But because each new test measures a slightly different variable, comparisons can be difficult to make.

"We can generate numbers *in vitro*," says Frazier, of CAAT. "The question is, what do those numbers mean?"

Moreover, says Dennis M. Stark, director of research and testing alternatives at Rockefeller University, with so many laboratories developing their own *in vitro* tests, "Any list [of standards] that comes out of a laboratory is going to look self-serving." So here, too, he says, the role of the federal agencies will be a critical one.

Stark says a recent meeting with Gary Flamm, the director of the FDA's office of toxicological sciences, left him convinced that the agency was serious about promoting *in vitro* alternatives. Flamm "seemed very interested in moving this thing from ground zero. He listened to us and seemed interested in getting things done."



A laboratory mouse getting a forced oral dose of a suspected toxin as part of the LD_{50} test.

However, Stark warns, "Companies are apt to keep doing the LD_{50} because of fear of litigation, even if the FDA doesn't insist." He has suggested that the FDA start accepting LD_{50} results using fewer animals whenever the data are supported by *in vitro* tests. "This would help reduce the number of animals being used, but it would still use some animals so that corporate lawyers would feel more secure," he says. In addition, such combinations of tests would move the *in vitro* validation process forward by adding to the database of *in vitro* results.

Ultimately, some argue, even regulatory revisions may be insufficient. Christopher Kelly of the National Testing Corporation in Palm Springs, Calif., a developer of *in vitro* toxicology tests, says that nothing short of congressional action is needed to ensure *in vitro*'s acceptance.

"Regulators only do what the law tells them to do," he says. "All in all, nothing is going to change until the law changes." He notes that one bill, HR 1635, now pending in Congress, would insist that if there is a valid *in vitro* test available, it must be used.

Others, including Spira, say that even such a law may not be strong enough. Unless specific reductions in the number of animal tests are spelled out, he says, with real penalties for failure to use *in vitro* tests, "creeping routinism" will ensure that unnecessary animal tests continue to prevail. □

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by copying the solution from a fellow student.

The answer is to give problems that require thought as well as manipulation, and to require the student to write out all the steps, just as was once done in high school geometry. It will also require the teacher or problem assistant to read the solution with care. This will entail more effort than is often currently devoted to teaching calculus, but the students deserve no less.

Harry H. Denman
Professor of Physics
Wayne State University
Detroit, Mich.

I was pleased to read of the "calculus reform" movement. I hope, however, that the movement does not stop at calculus and extends into high school and grade school. For many years math has been taught as if every student were a math major and could appreciate and understand its intrinsic beauty. In reality, math is a tool that is used to solve practical problems and has no other value to the vast majority of its students. The standard teaching approach, which stresses theoretical development, does not offer the student a tangible goal toward which the development is leading. Students generally have no idea where the math is taking them. They are just following directions.

If a new math concept were introduced to students by presenting a problem whose solution would be greatly aided by the new concept, then the development of the math toward that solution would have some meaning to them.

Math teaching is dominated by those who see and appreciate the pure beauty inherent in the subject. The students, however, do not share this vision, and so math has developed its fearful reputation. Mathematicians frequently lack the basic pragmatism toward the problem-solving role of math that others, such as engineers, have developed.

We are alienating our students from math at a time when its importance in our society is blossoming. Students need a better understanding of what math can really do for them.

Paul W. Dueweke
Palo Alto, Calif.

Ho hum. Another conference on the crisis in teaching college calculus. When will people face the truth? Little kids, learning at home, are able and eager to learn college math and science, if given chances to do so. But we don't offer them.

Many of the most interesting, important and useful features of science (nature) and its language, mathematics, are kept in a few college courses. Unless a person takes these courses, he or she never gets a chance to learn them. Moreover, they are given to prepare for careers in physical science or engineering. Of many tragic results, parents with training in these fields can't talk to their own children about them.

The above can be changed with materials to learn enjoyably "college level" math and science at home, at any time of life. Until we face these facts, and do constructive things about them, the futile conferences will continue.

Robert G. Hoffmann
Indianapolis, Ind.