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CHAPTER 1

History of the 3Rs in Toxicity Testing: From Russell and Burch to 21st Century Toxicology

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1.1 Introduction

Toxicity testing is an important part of the process of assessing the hazards, safety, or risk that chemicals and other substances pose to humans, animals, or the environment. The early toxicity tests that went on to enter routine use were developed in the first half of the 20th century. These included the LD50 test for acute systemic toxicity¹ and the Draize test for eye irritancy.² These procedures used vertebrate animals as test subjects – typically rodents in the LD50 test and rabbits in the Draize test.

As the new science of toxicology progressed, it continued to rely heavily on animals as test subjects. This was due largely to the rise of animal (mostly rodent) breeding for science in general, the virtual absence of more sophisticated ways of assessing toxicity, and the low status of animals in society.

While animal use in toxicology and the life sciences in general rose over the course of the early and mid-20th century, two parallel trends emerged. Concern

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for animal welfare was growing, and the life sciences were flourishing, with dramatic advances in knowledge and technique. It was in this context that the Universities Federation for Animal Welfare (UFAW), founded in England in 1926, made the fateful decision in the mid-1950s to undertake an ambitious survey of humane experimental techniques in animal-based experimentation throughout the life sciences.³ The project culminated in a pioneering book, *The Principles of Humane Experimental Technique* (hereinafter “*The Principles*”).⁴

The book’s authors – scientists William Russell and Rex Burch – proposed the 3Rs framework for making progress on both scientific and animal welfare fronts. Specifically, they advocated using scientific ingenuity to replace, reduce, and refine the use of animals wherever feasible without compromising scientific rigor. Russell and Burch’s extensive discussion of each of the 3Rs included numerous and diverse examples of each approach, drawing on their broad knowledge of the life sciences (and that of the experts they consulted).

“Refinement” referred to modifications in procedures that resulted in the animals experiencing less pain, distress, or discomfort. In discussing refinement, Russell and Burch considered a wide range of issues, including anesthesia, analgesia, euthanasia technique, injection sites, use of less sentient species, and adoption of less intense experimental procedures to induce stress. The scope of refinement eventually expanded beyond limiting negative effects and came to include enhancing animal welfare, such as through housing social animals in groups rather than individually, or enriching their cage environment with objects such as nesting material.⁵

“Reduction” referred to careful design and analysis of animal-based experiments so that fewer animals could be used. In this context, Russell and Burch discussed a variety of approaches, such as calculating the minimum group size(s) needed for a particular experiment, conducting testing sequentially rather than concurrently to exploit information learned in prior stages, and employing advanced experimental designs (e.g., blocking) that increased statistical power while using fewer animals. They also called for increased use of genetically uniform animals, or the offspring of crosses between two different in-bred lines, as a means of controlling inter-individual variation. And more generally, they argued that it is contrary to the spirit of reduction to waste animals on experiments that are poorly conceived, designed, or statistically analyzed.

Finally, “replacement” referred to ways to avoid using whole, sentient animals, by the use of: (i) non-animal approaches such as *in vitro* methods, microorganisms, ethical human studies, and computer simulation, (ii) experiments using invertebrates, or early stage vertebrate embryos, and (iii) anesthetized vertebrates. Over time, use of anesthetized vertebrates has come to be viewed as refinement rather than replacement.

In the 1960s and 1970s, animal protection organizations began to use the term “alternatives” for the 3Rs, especially replacement, as part of their challenge to the status quo. Thus the “alternatives” label proved to be more politically charged than did the “3Rs.”

The forceful championing of alternatives left some scientists uneasy.⁶ In response, they began to push back. In 1985, for example, Swiss scientist

W. H. Wiehe argued that “alternative methods are a fallacy” and that *in vivo* experiments are “irreplaceable.”⁷ Ironically, a 1978 book by the Research Defence Society (in England) had explicitly and approvingly used this term “alternatives” to refer to each of the 3Rs in a book entitled “Alternatives to Animal Experiments.”⁸

Yet a little more than a generation after Wiehe’s dismissive remark, following the 2007 publication of a US National Research Council (NRC) report on “Toxicity Testing in the 21st Century, A Vision and a Strategy,”⁹ prominent scientists were predicting the near elimination – if not the total replacement – of animal use in toxicity testing through the development of “21st Century Toxicology.” Melvin Andersen and Daniel Krewski (members of the committee that drafted the NRC report) noted that the report “envisions a not-so-distant future in which virtually all routine toxicity testing would be conducted in human cells or cell lines *in vitro*.”¹⁰ Key government scientists in the United States, led by Francis Collins, currently director of the National Institutes of Health (NIH), wrote in a 2008 policy forum of the prestigious journal *Science* that federal initiatives are “promoting the evolution of toxicology from a predominantly observational science at the level of disease-specific models *in vivo* to a predominantly predictive science focused on broad inclusion of target-specific, mechanism-based, biological observations *in vitro*.”¹¹ That same year Collins’ predecessor, Elias Zerhouni, had referred to these initiatives as the beginning of the end of animal testing.¹² In an 2011 editorial about the efforts of the US Food and Drug Administration (FDA) to modernize regulatory science, FDA Commissioner Margaret Hamburg wrote in *Science* that the agency is “working to eventually replace animal testing with a combination of *in silico* and *in vitro* approaches.”¹³

How have we gotten from Russell and Burch to the beginnings of 21st Century Toxicology? That journey will be the focus of this chapter.

1.1.1 Measuring 3Rs Activity: Our Approach

In our effort to trace the path from Russell and Burch to 21st Century Toxicology, we compile information on a wide range of 3Rs activities in the field of toxicology from 1959, when *The Principles* was published, to the present. In doing so, we want to supplement a conventional narrative approach to a historical review, which can be subjective in the choice of events and papers considered – and therefore in the interpretations offered – with more objective measures.

To that end, we begin with comprehensive citation and literature searches to trace the influence of Russell and Burch’s 3Rs framework and the prevalence of 3Rs-related research in toxicology over time, as revealed by patterns in the toxicological literature. When did Russell and Burch’s framework start influencing the field of toxicology? How relevant have the 3Rs been to toxicology research? These are the kind of questions we sought to answer.

We then present timelines of various 3Rs activities to inform our historical analysis. These activities include the founding of 3Rs organizations and centers,

the establishment of 3Rs funding sources, the enactment of animal welfare/alternatives laws, the founding of 3Rs journals and websites, the occurrence of 3Rs workshops and conferences, and other milestones.

Following this, we integrate the findings from the literature searches and timelines to briefly tell the story of the 3Rs in toxicology, framing the narrative around what we regard as four phases of activity:

- incubation (1959–1979),
- increasing acceptance and spread (1980–early 1990s),
- maturation (early 1990s–2007), and
- paradigm shift (2007–present).

We then look at measures of the impact of this 3Rs activity on toxicity testing, focusing on alternative methods that have been successfully validated and accepted for regulatory use and any impact of these methods on trends in animal use. We conclude with a section on remaining challenges to replacing animal use in toxicology.

A number of narrative histories of the 3Rs in toxicology or in the life sciences generally have been written previously.^{14–17} Similarly, although not necessarily focused on toxicology, a number of reviews have been written regarding refinement,^{18–20} reduction,^{21–23} and replacement,^{24–27} separately or in combinations.^{28,29} In our survey, we bring some of these earlier reviews up-to-date, focusing exclusively on toxicology and treating the 3Rs as a holistic framework, rather than covering each R thematically. We also explore literature searches as a source of additional historical insight and attempt to objectively assess the impact of the past 50-plus years of 3Rs activity in toxicology.

1.2 3Rs-Related Trends in the Toxicological Literature

Citation and literature searches can reveal historical patterns in the uptake and prevalence of 3Rs-related research in toxicology. To our knowledge, this approach has not been thoroughly investigated before. Some noteworthy findings are discussed here, and are incorporated in our discussion of historical phases of 3Rs activity (Section 1.4).

Our searches were designed specifically to capture toxicology-related papers that: (i) cite the book that launched the 3Rs framework (*The Principles*, both the 1959 original and a 1992 reprinting³⁰), (ii) cite the NRC report that proposed a paradigm shift to non-animal-based toxicity testing (*Toxicity Testing in the 21st Century*), and (iii) explicitly address one or more of the 3Rs or alternatives, based on the presence of selected 3Rs terminology, synonymous phrases, or database indexing terms. This last set of papers was further analyzed to explore the prevalence of key 3Rs topics, such as validation of new methods, based on the inclusion of relevant terminology in the paper's title, abstract, or keywords. (See Appendices A and B for further details of our search strategies.)

An analysis of citations in the toxicological literature to Russell and Burch's pioneering book and the NRC's seminal report is perhaps the most direct assessment of the influence of these works over time and the extent of discussion about the ideas they espoused. The literature searches for 3Rs-related publications, meanwhile, give a sense of the prevalence of "alternatives" work devoted to investigating, considering, or applying 3Rs concepts within the field of toxicology over the past 50-plus years, irrespective of whether or not Russell and Burch or *Toxicity Testing in the 21st Century* were explicitly cited.

According to our results, from 1959, when Russell and Burch's *The Principles* was first published, to 2011, the last year for which we have complete data, 438 publications in the toxicology/pharmacology literature cited their work.^a The search for toxicological papers addressing one or more of the 3Rs yielded nearly 3200 publications.

These findings indicate that far more papers discussed or applied replacement, reduction, refinement, or alternatives concepts than cited Russell and Burch's pioneering book. Perhaps one reason for this discrepancy, at least in recent years, is that authors take the 3Rs framework as a given, without the perceived need to reference its origin. As the 3Rs framework becomes increasingly integrated into toxicity testing programs and animal research policies (e.g., via the REACH program in Europe³¹), these efforts may act as other, more recent drivers for authors considering the question.

Notwithstanding the discrepancy in absolute numbers, the historical trend in toxicological publications citing *The Principles* (Figure 1.1) is similar to those addressing one or more of the 3Rs (Figure 1.2). There was a clear initial period of relative dormancy following publication of *The Principles* in 1959, lasting until the early 1980s, as revealed by the relative dearth of publications citing Russell and Burch or addressing the 3Rs. Citations and 3Rs papers began appearing regularly around 1980, with a fairly steady growth starting in the 1990s and a sharp uptick after 2007.

The dip in citations to *The Principles* starting in 2000 (Figure 1.1) is possibly an artifact of our search strategy, as we had augmented our search with two additional databases for the years prior to 2000 (see Appendix A for details). Removing the citations unique to those extra databases produces a more consistent upward trend (data not shown).

Parsing our universe of 3Rs-related papers from Figure 1.2 reveals finer-grained trends in the 3Rs literature. Figure 1.3, for example, shows that in the earlier years, a far greater percentage of 3Rs papers mentioned reduction (in the title, abstract, or keywords) than either refinement or replacement. This is surprising in that much of the reduction literature is generic – not specifically focused on toxicology or any other particular discipline. We suspect that some of these early toxicology papers were using "reduction" loosely to refer to diminishing animal use through either replacement or reduction.

^aWe emphasize that this represents just some of all the citations to Russell and Burch's book throughout the scientific literature during this period.

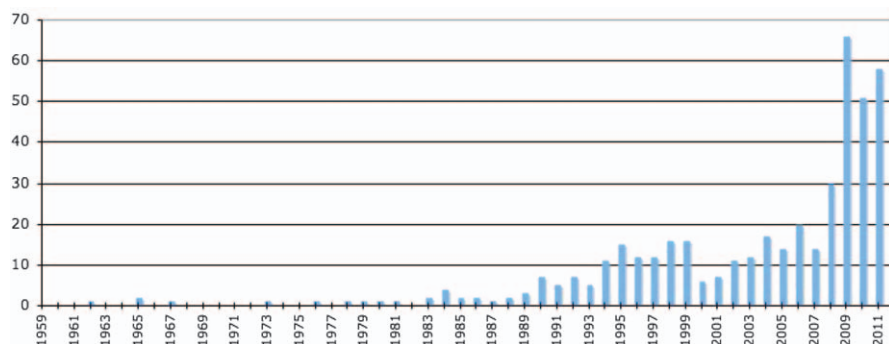


Figure 1.1 Citations to Russell and Burch's *The Principles of Humane Experimental Technique* (1959 and 1992 editions) by year (1959–2011), as identified by citation searches of Web of Science, BIOSIS, and SCOPUS databases through 2011, as well as SciSearch and Google Scholar databases through 1999, limited to the toxicological or pharmacological literature.

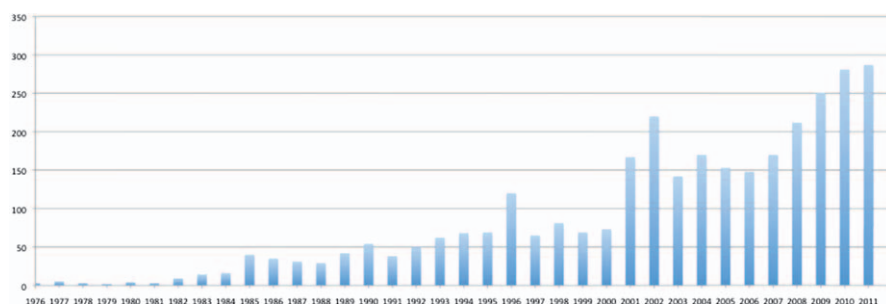


Figure 1.2 Publications related to the 3Rs and alternatives by year (1976–2011), as identified by literature searches using Embase and Ovid Medline databases and limited to the toxicological literature.

Over time, however, refinement and replacement papers became more prevalent, narrowing the gap between reduction papers on the one hand and refinement and replacement papers on the other. By 2011, approximately 40% of the papers we examined were related to reduction, 30% to replacement, and 20% to refinement. Removing those papers that mention all three Rs from the analysis does little to change this trend (data not shown). Similarly, inclusion of those papers found in the database searches using synonyms of “reduction” or “refinement” (e.g., “decreased use of animals,” “lessen pain,” etc.) does not substantively change the results, probably because these represent a very small percentage of papers in a given year (data not shown). (No searches were conducted using synonyms of “replacement.”)

Interestingly, papers that did not explicitly mention any of the 3Rs (replacement, reduction, or refinement) typically constituted the majority until the early 2000s. These papers were likely identified during our literature search

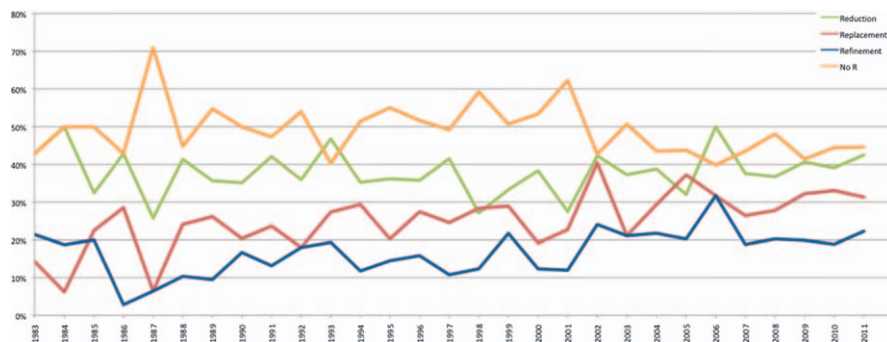


Figure 1.3 Percentage of 3Rs-related publications of those identified in Figure 1.2 mentioning “replac” (Replacement), “reduc” (Reduction), or “refin” (Refinement) in their title, abstract, or keywords (1983–2011). Some papers fall under multiple categories if they have multiple R terms. “No R” refers to those papers that have none of the R terms in the title, abstract, or keywords (e.g., papers that use the term “alternative” or “3Rs” instead).

owing to their use of the more general term “alternative” or “3Rs,” rather than a specific R term. During the 2000s, papers not mentioning any R became less frequent, and these papers made up an almost equal percentage as reduction papers by 2011.

Looking at our universe of 3Rs-related toxicology papers from Figure 1.2 in a different way, we determined the frequency with which certain key topics appeared in this collection. We made no attempt to be exhaustive, although we did choose a representative sampling of alternatives-related topics. These were as follows:

- “*In vitro*,” “cell culture,” and/or “tissue culture”: these terms broadly represent the most commonly used type of non-animal testing.
- “*In silico*” and/or “SAR” (Structure–Activity Relationships): these computer-based methods, including (Quantitative) Structure–Activity Relationships, generate toxicological predictions based on chemical properties.
- “Validation”: this is the process by which the relevance and reliability of methods are assessed for a particular purpose (see also Section 1.5.1). These are formal assessments of whether test methods (often new alternative methods) are fit for purpose and ready for consideration to be included in regulatory toxicology.
- “Testing strategies”: these are frameworks incorporating two or more types of testing. Commonly known as “integrated” or “intelligent” testing strategies, these efforts are often employed as a means of increasing testing efficiency and thereby limiting animal testing.
- “Humane endpoint”: a type of refinement in which an experiment is terminated at an earlier point, sparing animals unnecessary suffering without loss of experimental information.³²

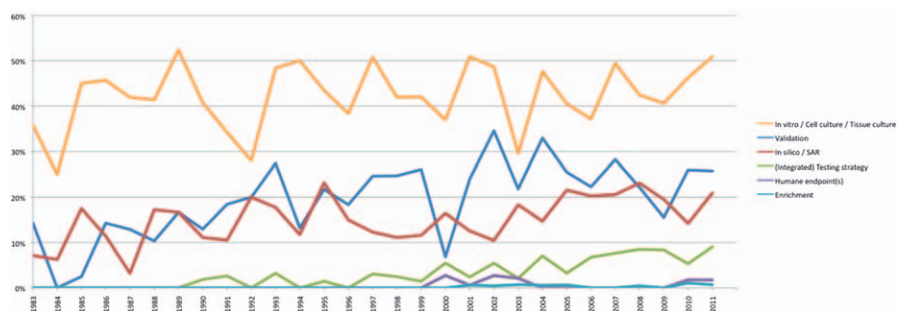


Figure 1.4 Percentage of 3Rs-related publications of those identified in Figure 1.2 mentioning select key topic phrases in their title, abstract, or keywords (1983–2011). Variants of key phrases were included when applicable, e.g., “validate” for Validation; “SAR,” “structure activity relation,” or “structure-activity relation” for (Q)SAR; and “testing strategy” for (Integrated) testing strategy.

- “Enrichment”: a type of refinement in which an animal’s living situation is enhanced through various means, including the provision of a social partner, nesting material, or food puzzles.³³

Papers mentioning “*in vitro*,” “cell culture,” and/or “tissue culture” in the title, abstract, or keywords consistently constituted 30–50% of the 3Rs papers over time (Figure 1.4). Other topics, such as “*in silico*” and/or “SAR” and, to a greater degree, “validation,” rose in prominence over the years. “Validation” represented a quarter to a third of the 3Rs papers most years from 1993–2011. The concept of (integrated) testing strategies first appeared in this literature in 1990 and increased noticeably in the 2000s. Other topics, such as “humane endpoint” and “enrichment” have a recent but minor presence in the 3Rs literature in toxicology.

Another important topic relevant to the history of the 3Rs in toxicology is “21st century toxicology” as exemplified in the 2007 NRC report on *Toxicity Testing in the 21st Century* (see also Sections 1.1, 1.3, and 1.4.4). This report has considerable relevance to replacement in toxicology. Interestingly, it was not the output of the 3Rs community per se but it was clearly the product of an era informed by Russell and Burch and the 3Rs approach, as well as by a broader concern for animal welfare and an appreciation for how far science and technology had advanced since the early days of animal testing.³⁴ We conducted a separate citation analysis on this report. From 2007, when *Toxicity Testing in the 21st Century* was published, to 2011, we identified 216 citations to it, indicating that the report has generated extensive discussion and implementation during the few years since its publication. Perhaps tellingly, citations to the report in the last two years have surpassed those to *The Principles* (Figure 1.5), and preliminary data indicate that the same is likely to be true in 2012.

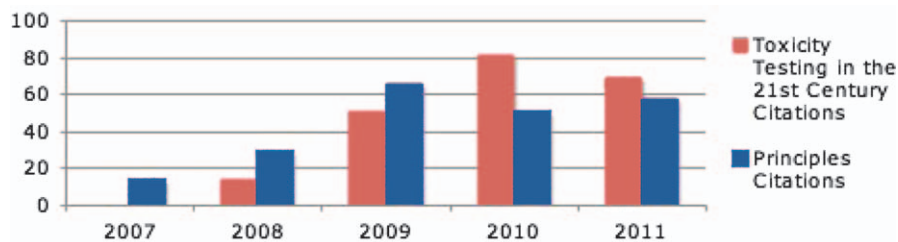


Figure 1.5 Citations to the National Research Council's *Toxicity Testing in the 21st Century: A Vision and a Strategy* (2007) by year (2007–2011), as identified by citation searches of Web of Science, BIOSIS, and SCOPUS databases through 2011 and limited to the toxicological and pharmacological literature, alongside citations to Russell and Burch's *The Principles of Humane Experimental Technique* from 2007–2011 (see Figure 1.1).

Our citation and literature searches provide insights regarding the influence of Russell and Burch and their 3Rs framework in toxicology, but they cannot be expected to reveal the whole story. For one thing, while our searches were comprehensive, they were not exhaustive. This, combined with other limitations in database searching, means we have likely underestimated the absolute number of citations and 3Rs papers. (For further discussion of the limitations of our approach, see Appendices A and B.)

In addition, our searches do not capture the 3Rs work done outside the field of toxicology but that has nevertheless influenced the work of toxicologists. During the early 1980s, for example, the work of Russell and Burch figured into more general questions of animal use, when papers related to the ethics and necessity of animal experimentation cropped up as the U.S. Congress debated and ultimately passed significant amendments to the Animal Welfare Act dealing with animals in research.

Many refinement and reduction papers have also been framed generally, rather than narrowly tailored to toxicology. “Humane endpoints” has been a hot topic in refinement for years, but is barely present in the toxicological literature. Toxicologists likely reference and make use of these sorts of papers, even if they are not indexed as toxicology papers.

1.3 Timelines of 3Rs Activities in Toxicology

Timelines can provide an event-based view of the development of the 3Rs in toxicology over time. We compiled 3Rs timelines that update those published previously^{14–16} and focus them primarily on toxicology. We also group events according to activity type rather than by time blocks to allow for an additional level of analysis. Unlike the literature searches in Section 1.2, the timelines are largely self-explanatory, so we simply provide an annotated list of them here as a prelude to weaving them into our discussion of historical phases of 3Rs activity in Section 1.4.

Our timelines are organized into the following activity types:

- *Early funding sources* (Table 1.1): the establishment of funding sources for “humane research,” which began in the 1960s, was one of the earliest manifestations of the impulse to promote alternatives research.
- *Early animal welfare/alternative laws* (Table 1.2): countries incorporated the 3Rs in their legislation beginning in the late 1970s.

Table 1.1 The establishment of some early funding sources for 3Rs research.

<i>Year</i>	<i>Event</i>	<i>Country</i>	<i>Note</i>	<i>Source</i>
1961	Humane Research Trust (initially the Lawson Tait Trust)	UK	The first research fund to support the scientific development of alternatives	http://www.humaneresearch.org.uk
1964	Swedish Fund for Research Without Animal Experiments	Sweden	Founded by Swedish Society Against Painful Experiments on Animals. First research grants awarded in 1971	http://www.forskautandjurforsok.se/in-english/
1973	Lord Dowding Fund for Humane Research	UK	A program of the National Anti-Vivisection Society	http://www.ldf.org.uk
1972	Felix Wankel Prize	Germany	A biennial award of up to 30,000 Euros	http://www.felix-wankel-forschungspreis.de
1979	The first government funding for alternatives	Sweden		Ref. 16
1981	Center for Alternatives to Animal Testing grants program	US		http://caat.jhsph.edu/programs/grants/
1985	International Foundation for Ethical Research	US	Launched by the New England Anti-Vivisection Society, currently affiliated with the National Anti-Vivisection Society	http://www.ifer.org
1989	Alternatives Research & Development Foundation	US	Affiliated with the American Anti-Vivisection Society	http://www.ardf-online.org

Table 1.2 The enactment of representative alternatives-related laws.

<i>Year</i>	<i>Event</i>	<i>Country</i>	<i>Note</i>
1977	Animal Protection Law includes a section on alternatives	The Netherlands	Has grown into a program that provides funding for alternatives research
1981	Legislation requires consideration of alternatives	Switzerland	
1985	Animal Welfare Act amendments call for consideration of alternatives	United States	
1986	European Community Directive 86/609	European Community	Requires member countries to develop legislation promoting the Three Rs
1986	Laws requiring consideration of alternatives in animal research	Germany	
1992	Cosmetic Directive bans marketing of cosmetics tested on animals after Jan. 1, 1998	European Union	A decision on the ban is later postponed until June 30, 2000
1994	The 1993 National Institutes of Health Revitalization Act leads to the establishment of ICCVAM	United States	
2000	ICCVAM Authorization Act	United States	Strengthened ICCVAM's status and mandate
2006	REACH mandates that chemical manufacturers or importers submit safety information	European Union	Articles 1 and 13 have pro-alternatives language

Abbreviations: ICCVAM: Interagency Coordinating Committee on the Validation of Alternative Methods; REACH: Registration, Evaluation, Authorisation and Restriction of Chemicals. Source: Ref. 16.

- *Journals and websites* (Table 1.3): several journals and websites devoted to alternative methods were founded, beginning in the early 1970s.
- *Early workshops and conferences* (Table 1.4): several conferences devoted to alternative methods were organized beginning in the mid-1970s, including ones that became part of a series, such as the Center for Alternatives to Animal Testing (CAAT) symposia, as well as the ongoing Linz conferences and World Congresses on Alternatives and Animal Use in the Life Sciences.
- *Alternatives organizations and centers* (Table 1.5): an impressive number of organizations and centers dedicated to the 3Rs were founded over the years. Among the most prominent are the Fund for the Replacement of Animals in Medical Experiments or FRAME (1969), CAAT (1981),

Table 1.3 The founding of some representative journals and websites devoted to the 3Rs or non-animal methods.

Year	Journal/Website	Note	Source
1973	The journal <i>Alternatives to Laboratory Animals (ATLA)</i>	Founded by Fund for the Replacement of Animals in Medical Experiments	http://www.frame.org.uk
1984	The journal <i>ALTEX</i> , devoted to the 3Rs of animal experimentation	Founded by Animalfree Research, now issued by Swiss Society ALTEX Edition	http://www.animalfree-research.org
1986	The journal <i>Toxicology In Vitro</i>		http://www.journals.elsevier.com/toxicology-in-vitro/ http://www.asas.or.jp
1990	The journal <i>Alternatives to Animal Testing and Experimentation (AATEX)</i>	Published by the Japanese Society for Alternatives to Animal Experimentation	
1996	<i>Altweb</i> , a website devoted to the Three Rs in the life sciences	Founded by the Center for Alternatives to Animal Testing and numerous partners	http://altweb.jhsph.edu
2007	<i>AltTox</i> , a website devoted to non-animal alternatives in toxicology	Founded by the Humane Society of the United States and the Procter and Gamble Company	http://www.alttox.org

Center of the Documentation and Evaluation of Alternative Methods to Animal Experimentation [better known by its German acronym, ZEBET] (1989), European Centre for Alternatives to Animal Methods or ECVAM (1992), the US-based Interagency Coordinating Committee on the Validation of Alternative Methods or ICCVAM (1994), and Japanese Center of the Validation of Alternative Methods or JaCVAM (2005).

- *Developments related to pathway-based testing* (Table 1.6): the publication of the US NRC report in 2007 on *Toxicity Testing in the 21st Century, A Vision and a Strategy*⁹ revolutionized thinking about the future of toxicity testing (see also Sections 1.1, 1.2, and 1.4.4). Its emphasis on upstream, pathway-based testing has led to – or provided intellectual backing for – a number of important efforts with dramatic implications for replacing animal use in toxicology, including the U.S. Environmental Protection Agency's ToxCast program and the multi-agency Tox21 program.

These timelines depict representative events and are not necessarily exhaustive. Particular attention was paid to early developments; for some

Table 1.4 Representative early workshops and conferences on the 3Rs and alternatives.

<i>Year</i>	<i>Event</i>	<i>Note</i>	<i>Source</i>
1975	The National Academy of Sciences holds the US's first major scientific meeting on alternatives	Topic: "The Future of Animals, Cells, Models, and Systems in Research, Development, Education, and Testing"	Ref. 57
1978	FRAME hosts Europe's first big scientific meeting on alternatives	Topics: "Alternatives in Drug Development and Testing"	http://www.frame.org.uk/page.php?pg_id=42
1982	CAAT's first symposium	Center for Alternatives to Animal Testing	Ref. 14
1982	FRAME hosts "Animals and Alternatives in Toxicity Testing" conference	The FRAME Toxicity Committee presents its first report	http://www.frame.org.uk/page.php?pg_id=42
1988	The first meeting of the Industrial In Vitro Toxicology Group	Corporate toxicologists applying in vitro methods	Ref. 14
1991	First conference in Linz, Austria, later the European Congresses on Alternatives to Animal Testing	Initially organized by animal protectionists, then by MEGAT/EUSAAT and later joined by ZET (Austrian alternatives platform)	Horst Spielmann (personal communication)
1993	The first World Congress on Alternatives and Animal Use	Subsequent WCs held in 1996, 1999, 2002, 2005, 2007, 2009, and 2011.	Ref. 58
1996	OECD holds a workshop on validation and regulatory acceptance	Aim: to develop internationally harmonized criteria	Ref. 59

Abbreviations: CAAT: Center for Alternatives to Animal Testing; FRAME: Fund for the Replacement of Animals in Medical Experiments; MEGAT: Middle European Society for Alternatives to Animal Testing; EUSAAT: the European Society for Alternatives to Animal Testing; OECD: Organisation for Economic Co-operation and Development.

activities, recent developments were too numerous to be included in their entirety.

1.4 Phases in the History of the 3Rs in Toxicology

Taking together the results of our literature searches (introduced in Section 1.2) and our timelines of important events (introduced in Section 1.3), we find it helpful to view the history of the 3Rs from Russell and Burch to 21st Century Toxicology as a progression through four phases: incubation, increasing acceptance, maturation, and paradigm shift. We divide the following historical narrative into these (somewhat overlapping) phases simply as a heuristic device to aid interpretation and understanding. We do not intend the phases to be

Table 1.5 The founding of representative alternatives organizations and centers.

<i>Year</i>	<i>Event</i>	<i>Country</i>	<i>Source</i>
1969	Fund for the Replacement of Animals in Medical Experiments (FRAME)	United Kingdom	http://www.frame.org.uk
1981	Center for Alternatives to Animal Testing (CAAT)	United States	http://caat.jhsph.edu
1985	The European Research Group into Alternatives to Toxicity Testing (ERGATT)	Europe	
1986	The Dutch Alternatives to Animal Experiments Platform	Netherlands	http://oslovet.norecopa.no/platform/report/ecopaplatforms.pdf
1986	Foundation for the Promotion of Alternate and Complementary Methods to Reduce Animal Testing (SET)	Germany	http://www.stiftung-set.de/index.php?id=3&L=1
1987	3R Research Foundation	Switzerland	http://www.forschung3r.ch/index_en.html
1989	Center for the Documentation and Evaluation of Alternative Methods to Animal Experimentation (ZEBET)	Germany	http://www.bfr.bund.de/en/zebet-58194.html
1992	European Centre for the Validation of Alternative Methods (ECVAM)	Europe	http://ec.europa.eu/dgs/jrc/downloads/jrc_press_animal_ecvam_overview.pdf
1994	Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)	United States	http://iccvam.niehs.nih.gov
1994	Netherlands Centre for Alternatives to Animal Use (NCA), now National Knowledge Centre on Alternatives (NKCA)	Netherlands	http://www.nca-nl.org
1996	Prince Laurent Foundation	Belgium	http://www.fondation-prince-laurent.be
1997	Institute for In Vitro Sciences (IIVS)	United States	http://www.iivs.org
1999	Spanish National Platform on Alternatives (REMA)	Spain	http://www.remanet.net/noticias/articulos/ncawesletter22032007.htm

2000	European Consensus Platform for Alternatives (ECOPA)	Europe	http://www.ecopa.eu
2004	National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs)	United Kingdom	http://www.nc3rs.org.uk
2004	International Centre for Alternatives in Research and Education (I-CARE)	India	http://www.icare-worldwide.org/india/doerenkamp-center.html
2005	European Partnership for Alternative Approaches to Animal Testing (EPAA)	Europe	http://ec.europa.eu/enterprise/epaa/index_en.htm
2005	Japanese Center for the Validation of Alternative Methods (JaCVAM)	Japan	http://jacvam.jp/en/
2007	Norwegian Consensus Platform for Replacement, Reduction and Refinement of Animal Experiments (Norecopa)	Norway	http://www.norecopa.no
2007	French Platform for the Development of Alternative Methods in Animal Testing (FRANCOPA)	France	http://www.francoipa.fr
2007	Centre for Advanced Research & Development of Alternative Methods (CARDAM)	Belgium	http://www.cardam.eu/
2008	Finnish Centre for Alternative Methods (FICAM)	Finland	http://ficam.fi
2009	Center for Alternatives to Animal Testing (CAAT) – Europe	Germany	http://cms.uni-konstanz.de/leist/caat-europe/
2010	South Korean Centre for the Validation of Alternative Methods (KoCVAM)	South Korea	http://ihcp.jrc.ec.europa.eu/glossary/kocvam
2010	American Society for Cellular and Computational Toxicology (ASCCT)	United States	http://ascctox.org/index.cfm
2011	Brazilian Center for Validation of Alternative Methods (BraCVAM)	Brazil	http://www.altex.ch/en/index.html?id=17&ncat=1&nid=192

Note: For additional listings, see http://www.frame.org.uk/page.php?pg_id=263

Table 1.6 Representative developments related to pathway-based testing as exemplified by the National Research Council report on *Toxicity Testing in the 21st Century*.

<i>Year</i>	<i>Event</i>	<i>Note</i>	<i>Source</i>
2007	<i>Toxicity Testing in the 21st Century: A Vision and a Strategy</i> published	A US National Research Council report commissioned by the US EPA	http://www.nap.edu/openbook.php?record_id=11970
2007	US government launches Tox21 program, including the EPA component, ToxCast	A partnership among several federal agencies	http://epa.gov/ncct/Tox21/ http://www.epa.gov/ncct/toxcast/
2010	Human Toxicology Project Consortium holds a conference on accelerating the transition to pathway-based testing	The Consortium is a multi-stakeholder effort	http://htpconsortium.files.wordpress.com/2012/09/stephenstoxscifeb2012.pdf
2010	AXLR8 established	Coordinates R&D to accelerate the transition to pathway-based testing	http://axlr8.eu
2011	SEURAT-1 multi-million Euro program established	“Safety Evaluation Ultimately Replacing Animal Testing”	http://www.seurat-1.eu
2011	Human Toxome Project established at CAAT	Mapping pathways of toxicity	http://www.altex.ch/en/index.html?id=50&iid=123&aid=1
2012	The Hamner Institutes for Health Sciences begins case study approaches to implementing the NRC vision	Designing pathway assays for use in risk assessment	http://www.thehamner.org
2012	OECD establishes Adverse Outcome Pathways (AOPs) as a basic principle of the Test Guidelines Programme	Issues Draft Guidance: Proposal for a Template and Guidance on Developing and Assessing the Completeness of [AOPs]	http://www.oecd.org/env/ehs/testing/49963554.pdf

taken too literally or to represent the only meaningful way to designate eras within this history.

1.4.1 Incubation (1959–1979)

As noted in Section 1.2, there is a clear time-lag between the publication of *The Principles* in 1959 and the emergence of publications in the toxicological literature that either cite this book (Figure 1.1) or mention the 3Rs concepts it described (Figure 1.2). This delayed uptake is consistent with the relative paucity of noteworthy events during this period, as revealed by the timelines (Section 1.3). Nonetheless, the main and enduring events during this phase were: (1) the establishment of several early funding sources for 3Rs research, the first of which was the (British) Humane Research Trust (Table 1.1), and (2) the founding of the alternatives center FRAME (Table 1.5) and its journal *ATLA* (Table 1.3). These early events occurred primarily in Great Britain, fittingly the home country of Russell and Burch and UFAW – the organization that launched their project.

These findings are consistent with the common understanding that, for toxicology and the life sciences in general, Russell and Burch figuratively wandered in the wilderness for decades before their book got the attention it deserved.^{14,16,35} In hindsight, there are perhaps many reasons for this. For example, after the book was published, William Russell, Rex Burch, and UFAW each quickly moved on to other challenges, largely leaving it to others to take up the ideas in the book.

Another possible reason for the lag in attention to *The Principles* and its 3Rs framework in toxicology may have been the early appearance of the book relative to the emergence of the field of toxicology as a scientific discipline. *The Principles* was published in 1959 before toxicological societies had even been established in the United States (1961) and Britain (1979). It also appeared early in the history of laboratory animal science, the discipline that emerged to address issues related to the care, management, and use of laboratory animals. In the United States, the first Guide for the Care and Use of Laboratory Animals was not published until 1963.^b

1.4.2 Increasing Acceptance and Spread (1980–early 1990s)

A second phase in the history of 3Rs activity in toxicology, roughly from 1980 through the early 1990s, is characterized by rising attention to the 3Rs, as evidenced both by increasing reference to *The Principles* (Figure 1.1), a greater number of 3Rs-related publications (Figure 1.2), and a clustering of notable developments.

Specifically, what we are characterizing as the increasing acceptance and spread of the 3Rs approach in Europe and North America are variously reflected in the establishment of alternatives centers such as CAAT and ZEBET

^b<http://www.aalas.org/association/history.aspx#Timeline>

(Table 1.5), the incorporation of the 3Rs framework in legislation such as the European Union legislation governing animal experimentation (EC 86/609) (Table 1.2), the founding of journals such as *ALTEX* dedicated to the subject (Table 1.3), and the organization of conferences such as FRAME's "Animals and Alternatives in Toxicity Testing" conference and a series of conferences organized by CAAT, each of which resulted in an edited volume of proceedings (Table 1.4).³⁶ In the United States, another noteworthy milestone in the increasing acceptance of the 3Rs approach was the 1986 publication of the Congressional Office of Technology Assessment's lengthy report on *Alternatives to Animal Use in Research, Testing, and Education*, which included two chapters on toxicity testing.³⁷

A key driver of the increasing acceptance of the alternatives approach, especially in toxicology, was the emergence of the animal rights movement and its criticism of animal experimentation, particularly procedures such as the Draize eye irritancy test that were used to assess cosmetics and other consumer products.⁶ In the United States, such criticism led Revlon to fund an alternatives research program at Rockefeller University and the Cosmetics Toiletries and Fragrance Association to establish CAAT.³⁸ Animal advocacy also led to a general expansion of funding sources for alternative methods, and helped to create the political climate that led to the incorporation of alternatives provisions in federal legislation such as the Animal Welfare Act and the ICCVAM Authorization Act (Table 1.2).

1.4.3 Maturation (early 1990s–2007)

A third phase in the history of 3Rs activity in toxicology, from roughly the early 1990s until 2007, was one of maturation of the field, characterized by continuing growth in publication of 3Rs papers (Figure 1.2) and citations to *The Principles* (Figure 1.1), and the founding of over a dozen alternatives centers (Table 1.5). Also noteworthy was the considerable activity around validation principles and processes (see also Section 1.5.1), with the emergence of national or regional validation centers in the European Union, the United States, and Asia (Table 1.5). Not surprisingly, then, the topic of validation was also increasingly prominent in the 3Rs literature (Figure 1.4), and dozens of alternative tests were validated (see Section 1.5.1), thanks in part to the work of ZEBET, ECVAM, ICCVAM, and JaCVAM.

During this period, the aim of one-to-one replacement of an animal test with an alternative test began to slowly give way to an appreciation of the value of integrated testing strategies, especially for challenging endpoints such as eye irritation and chronic systemic toxicity. This transition is reflected in the increasing representation of (integrated) testing strategy papers in the 3Rs literature (Figure 1.4). Nonetheless, the historical animal tests were, by and large, still assumed to represent the benchmark against which the performance of alternative tests and strategies were to be judged.

Given that 3Rs work was becoming increasingly international, the organization of the first World Congress on Alternatives and Animal Use in the Life

Sciences was a notable early event of this period. While the first major meetings on alternative methods began in the 1970s (Table 1.4), it was the World Congresses, initiated in 1993 by CAAT and reconvened every 2–3 years to the present, that gave those committed to the 3Rs a sense of community. Those meetings, which are devoted in large measure to toxicology issues, have drawn several hundred to over one thousand participants – scientists, administrators, regulators, funders, or animal advocates – from dozens of countries, and have been held in Europe, North America, and Asia.

International efforts on alternative methods have also been furthered by the work of the Organisation for Economic Co-operation and Development (OECD), an economic alliance of over 30 developed countries. These countries participate in the OECD's influential test guidelines program, which issues harmonized test guidelines and guidance documents, develops and validates test methods, coordinates testing programs, and encourages mutual acceptance of data generated using its approved guidelines.^c

1.4.4 Paradigm Shift (2007–present)

In many respects we are still in the maturation phase. However, a tipping point was reached in 2007 that leads us to designate a fourth (and current) era, one of a paradigm shift. The precipitating event was the publication of the NRC report on *Toxicity Testing in the 21st Century: A Vision and a Strategy* (Table 1.6; see also Sections 1.1 to 1.3). This report, commissioned by the U.S. EPA, gave us the phrase “21st Century Toxicology” with its emphasis on testing that is *in vitro*, focused largely on human biology, based on upstream biological pathways and perturbations to normal processes, often characterized using high-throughput methodology and supplemented with computational approaches.

In this framework, the focus is no longer on predicting apical endpoints in high-dose animal studies, such as tumors or death. Rather, the NRC report proposes developing assays that detect perturbations to fundamental biological pathways (e.g., DNA synthesis and repair) that would typically lead to adverse phenotypic outcomes. Precise predictions of those outcomes would be secondary to identifying upstream perturbations to be avoided. The NRC vision was proposed as a long-term transformation, and in the early years, any pathway-based testing would need to be heavily complemented by “targeted testing,” which would be mostly *in vivo*. Even such supplemental testing could move towards *in vitro* systems – in this case systems of a more complex and integrated nature (e.g., “organs on a chip”) – prior to the envisioned complete (or near complete) transition to pathway-based testing.

The proposed pathway-based framework was not the work product of the mainstream 3Rs community, although one of the charges to the NRC committee that developed the proposal was to consider ways to reduce animal testing, which itself is a reflection of the penetration of 3Rs/animal welfare ideas throughout the toxicology community. Not surprisingly, the proposal has been

^c<http://www.oecd.org/env/ehs/testing/>

embraced by the 3Rs community and seen as consistent with earlier calls for *in vitro* approaches and criticisms of animal testing.^{34,39}

Toxicity Testing in the 21st Century gained immediate recognition in the toxicological literature (Figure 1.5). Interestingly, citations to the report surpassed citations to *The Principles* in 2010 and 2011. The report perhaps also spurred the significant uptick in 3Rs-related toxicology papers since 2007.

A number of efforts have emerged seeking to promote “21st century,” pathway-based testing (Table 1.6). These include research and development programs on a large-scale in the United States (ToxCast and Tox21) and in the EU (e.g., SEURAT) and smaller scale efforts led by CAAT and the Hamner Institutes for Health Sciences. On the policy level, the Human Toxicology Project Consortium in the United States and the AXLR8 project in the European Union have sought to promote 21st century toxicology by assessing gaps in current R&D efforts and spurring needed efforts.

1.5 Impact Assessment of 3Rs Activity

To this point in the analysis, we have looked at the history of 3Rs activity in toxicology as revealed by patterns in the toxicological literature and by various timelines. So what difference has all this activity made in regulatory toxicity testing? We will examine this from two perspectives.

First, we look at alternative tests that have successfully gone through the process of validation and regulatory acceptance. Second, we assess whether these successes have had any discernible impact on overall trends in the use of animals in toxicology.

1.5.1 Validation and Acceptance Status of Alternatives

In vitro and other alternative tests have a long history of use in corporate decision-making about chemical safety and product formulation.⁴⁰ However, for many years such testing was not necessarily considered definitive in the regulatory context. Corporations would often follow up on their alternative testing with the historical animal-based methods. Validation – the formal assessment of the relevance and reliability of a test method for a particular purpose⁴¹ – came to be considered a prerequisite for regulatory use of alternatives.⁴²

Moreover, validation needed to be followed by a declaration of regulatory acceptance by the relevant government agencies, as a way of encouraging industry to use the validated tests and submit data based on them. Indeed, the need for successful validation and regulatory acceptance are written into U.S. law through the ICCVAM Authorization Act of 2000.^d

New and modified assays that have been validated and accepted for regulatory use are listed in Table 1.7. A number of patterns can be discerned. First, most of the assessments of validation status and regulatory acceptance have occurred since 2000, following the establishment of key alternatives centers

^dhttp://iccvam.niehs.nih.gov/docs/about_docs/PL106545.pdf

Table 1.7 Alternative test methods and testing strategies: Their validation and regulatory acceptance status.

	Full (✓) or Partial (✓*) Replacement ^a	Reduction	Refinement	Validation Status	Regulatory Acceptance
Aquatic Toxicity					
Upper threshold concentration step-down approach		✓		2006 (ESAC)	2010 (OECD) ^b
Acute Systemic Toxicity (Oral)					
Up-and-down procedure		✓		2001 (ICCVAM)	2006 (OECD)
Normal human keratinocyte neutral red uptake (NHK NRU) assay	✓*			2006 (ICCVAM)	2010 (OECD)
Balb/c 3T3 NRU assay	✓*			2006 (ICCVAM)	2010 (OECD)
Acute toxic class method		✓		2007 (ESAC)	2001 (OECD)
Fixed dose procedure		✓	✓	2007 (ESAC)	2001 (OECD)
Acute Systemic Toxicity (Inhalation)					
Acute toxic class method		✓			2009 (OECD)
Fixed concentration procedure		✓	✓		draft (OECD)
Carcinogenicity (Non-genotoxicity)					
Cell transformation assays	✓*			2012 (ECVAM)	draft (OECD)
Chronic Toxicity					
Removal of 1 year dog study for pesticides		✓		2006 (ESAC)	Revised US EPA Pesticide Data Requirements
Dermal Penetration					
<i>In vitro</i> skin absorption methods	✓* ^c			2002 (OECD)	2004 (OECD)
Endocrine Active Substances					
Androgen receptor binding assay (rat prostate cytosol)	Ex vivo*				2009 (EPA)
Aromatase inhibition assay (human recombinant)	✓*				2009 (EPA)
Estrogen receptor (ER)-alpha transcriptional activation assay for estrogen agonists (STTA)	✓*			OECD/EPA	2009 (OECD)

Table 1.7 (Continued)

	Full (✓) or Partial (✓*) Replacement ^a	Reduction	Refinement	Validation Status	Regulatory Acceptance
Estrogen receptor binding assay rat uterine cytosol (ER-RUC)	✓*(Ex vivo)				2009 (EPA)
H295R steroidogenesis assay	✓*			OECD/EPA	2009 (EPA), 2011 (OECD)
US EPA Tier 1 Screening Battery	✓*	✓			2009 (EPA)
BG1Luc ER TA test method for estrogen agonists and antagonists	✓*			2012 (ICCVAM)	draft (OECD)
Eye Corrosion					
Bovine corneal opacity permeability (BCOP) test	✓*			2007 (ICCVAM)	2009 (OECD)
Isolated chicken eye (ICE) test	✓*			2007 (ICCVAM)	2009 (OECD)
Cytosensor Microphysiometer modified (cytotoxicity/cell-based assay)	✓*			2009 (ESAC)	2010 (draft OECD)
Fluorescein Leakage (cytotoxicity/cell-based assay)	✓*			2009 (ESAC)	2010 (draft OECD)
Hen's egg test – chorioallantoic membrane (HET-CAM)	✓*				EU Competent Authorities for Dangerous Substances Directive
Isolated rabbit eye test	✓*				EU Competent Authorities for Dangerous Substances Directive
Routine use of topical anesthetics, systemic analgesics, and humane endpoints			✓	2009 (ICCVAM)	draft (OECD)
Eye Irritation					
Cytosensor Microphysiometer modified (cytotoxicity/cell-function based <i>in vitro</i> assay)	✓*			2009 (ESAC)	2010 (OECD draft)
Rabbit low-volume eye test (LVET)			✓	2009 (ESAC)	

Routine use of topical anesthetics, systemic analgesics, and humane endpoints		✓		2009 (ICCVAM)	2012 (Expected)
Genotoxicity					
<i>In vitro</i> sister chromatid exchange test	✓*				1986 (OECD)
<i>In vitro</i> unscheduled DNA synthesis test	✓*				1986 (OECD)
<i>Saccharomyces cerevisiae</i> gene mutation assay	✓*				1986 (OECD)
<i>Saccharomyces cerevisiae</i> mitotic recombination assay	✓*				1986 (OECD)
Bacterial reverse mutation (Ames) test	✓*				1997 (OECD)
<i>In vitro</i> mammalian cell micronucleus test	✓*			2006 (ESAC)	2012 (OECD)
Hematotoxicity: Acute Neutropenia					
Colony-forming unit granulocyte macrophage (CFU-GM) assay	✓*			2006 (ESAC)	Submitted to EMA
Immunotoxicity/Skin Sensitization					
Local lymph node assay (LLNA)		✓	✓	1999 (ICCVAM)	2002 (OECD)
Reduced LLNA: rLLNA		✓	✓	2007 (ESAC)	2010 (OECD)
Nonradiolabelled LLNA: DA		✓	✓	2008 (JaCVAM)	2010 (OECD)
Nonradiolabelled LLNA: BrdU-ELISA		✓	✓	2009 (ICCVAM)	2010 (OECD)
LLNA for potency characterization		✓	✓	2011 (ICCVAM)	2009 (UN GHS)
Phototoxicity					
3T3 Neutral Red Uptake Phototoxicity Test	✓			1997 (ESAC)	2004 (OECD)
3T3 NRU Phototoxicity Test: Application to UV filter chemicals	✓			1998 (ESAC)	2004 (OECD)
Pyrogenicity					
Human whole blood IL-1	✓*			2006 (ESAC)	EMA
Human whole blood IL-6	✓*			2006 (ESAC)	EMA
Human cryopreserved whole blood IL-1	✓*			2006 (ESAC)	EMA
PBMC IL-6	✓*			2006 (ESAC)	EMA
MM6 IL-6	✓*			2006 (ESAC)	EMA
Limulus amoebocyte lysate (LAL) test	✓*				European Pharmacopeia
Reproductive & Developmental Toxicity					
Embryonic stem cell test for embryotoxicity	✓*			2002 (ESAC)	OECD draft

Table 1.7 (Continued)

	Full (✓) or Partial (✓*) Replacement ^a	Reduction	Refinement	Validation Status	Regulatory Acceptance
Micromass embryotoxicity assay	✓*			2002 (ESAC)	
Whole rat embryotoxicity assay	✓*			2002 (ESAC)	
Extended one-generation reproductive toxicity study		✓			2011 (OECD)
Skin Corrosion					
EpiSkin [®] human skin model	✓			1998 (ESAC)	2004 (OECD)
Rat skin transcutaneous electrical resistance (TER) assay	✓			1998 (ESAC)	2004 (OECD)
Corrositex [®] noncellular membrane	✓			1999 (ICCVAM)	2006 (OECD)
EpiDerm [™] human skin model	✓			2000 (ESAC)	2004 (OECD)
SkinEthic [™] human skin model	✓			2006 (ESAC)	2004 (OECD)
Vitrolife-Skin human reconstructed epidermis	✓			2008 (JaCVAM)	2004 (OECD)
EST-1000 human reconstructed epidermis	✓			2009 (ESAC)	2004 (OECD)
Skin Irritation					
EpiSkin [®] skin irritation test (with MTT reduction)	✓			2007 (ESAC)	2010 (OECD)
EpiDerm [™] skin irritation test (with MTT reduction)	✓			2007 (ESAC)	EU test method B.46 in COM regulation 440/2008/EC
EpiDerm [™] SIT model (EPI-200)	✓			2008 (ESAC)	2010 (OECD)
SkinEthic RHE model	✓			2008 (ESAC)	2010 (OECD)

^aWhether or not a test method is a full or partial replacement is not always unambiguous.

^bThe upper threshold concentration step-down approach was issued as a guidance document because consensus could not be reached.

^cThe *in vitro* skin absorption methods do not apply to mixtures/formulations.

Abbreviations: EMA: European Medicines Agency; EPA: US Environmental Protection Agency; ESAC: ECVAM Scientific Advisory Committee; GHS: Globally Harmonized System of Classification and Labelling of Chemicals; ICCVAM: US Interagency Coordinating Committee on the Validation of Alternative Methods; JaCVAM: Japanese Center for the Validation of Alternative Methods; OECD: Organisation for Economic Co-operation and Development.

Adapted from <http://alttox.org/ttrc/validation-ra/validated-ra-methods.html>, last updated July 10, 2012.

(Table 1.5) and the development of the principles and procedures of validation and regulatory acceptance (see Section 1.4.3). Second, the bulk of this effort has been invested in replacement alternatives (full or partial), with acute systemic toxicity and skin sensitization being notable exceptions. This activity has been driven, in part, by the ban on animal testing for cosmetic ingredients, pursuant to the European Cosmetics Directive.

Much of what we might term “toxicological space” has been touched by alternative methods. This is especially true for acute toxicity endpoints, where replacement alternatives have become available for skin penetration, skin corrosion, skin irritation, and phototoxicity. However, the challenge of replacing animal use for chronic endpoints is much more formidable.²⁴ One prominent effort addressing the challenge of alternatives to chronic toxicity testing is the SEURAT program, a multi-million Euro partnership between the cosmetics industry and the European Union.^e A roadmap for replacing animals in chronic and systemic toxicity testing has been published recently.²⁵

Of course, validation and regulatory acceptance of new methods do not necessarily ensure full implementation of those methods in all cases, so the degree of implementation and any barriers to implementation would need to be addressed in a more definitive analysis of the impact of alternative methods on toxicology.^{43–45}

What would Russell and Burch themselves have made of the record of achievement as reflected in Table 1.7? Rex Burch died in 1996 and William Russell in 2006, but their writings and statements towards the end of their lives suggest their pride in what their pioneering book set in motion.^{3,46} Of course, much remains to be done, with formidable challenges ahead (see Section 1.6).

1.5.2 Historical Trends in Animal Use in Toxicology

Both reduction and replacement alternatives should result in fewer animals being used. However, animal use in any scientific field can be influenced by a host of other factors, including whether overall activity in that field is expanding or contracting over time. An expanding field could mask any gains from reduction and replacement alternatives, whereas a field in decline could exaggerate the perceived impact of such alternatives.

Given the trends we have seen in the history of the 3Rs in toxicology, we would expect the largest impact of the 3Rs on animal use in this field to be apparent since the beginning of the 2000s, following the establishment of the process of validation and of the validation centers themselves, as well as the actual validation and regulatory acceptance of individual tests (see Sections 1.4.3 and 1.5.1). Even prior to the validation and regulatory acceptance of alternatives, a general sensitivity to animal welfare and the 3Rs approach may have led to greater scrutiny of animal use in toxicology and subsequent reductions in animal numbers, especially in pre-regulatory toxicology. On the

^e<http://www.seurat-1.eu>

other hand, animal testing inevitably increases over the short term when new testing programs are launched (e.g., REACH), new endpoints are developed (e.g., endocrine disruption), and new types of chemicals are commercialized (e.g., nanoparticles).

Reports from several countries provide evidence that overall animal use (for any purpose, not just for toxicology) declined during the last quarter of the 20th century (Andrew Rowan, personal communication). To analyze international trends in animal use in toxicology, data need to be gathered in a way that allows comparison across countries. The best source for such data comes from the EU. Consistent statistics on animal use in toxicology began to be aggregated across EU member states (nations) in 1999, and since then have been compiled every three years, with 2008 the most recent compilation. These statistics do not allow us to assess the impact of early 3Rs activity, but they do cover the years when we would expect to see an impact from the development and validation of alternatives (see Sections 1.4.3 and 1.5.1).

We combined the statistics on numbers of animals used for toxicology with statistics on numbers of animals used for the production and quality control of products and devices for human medicine, dentistry, and veterinary medicine. We controlled for the fact that the number of EU member states has grown over the years in question by assessing data from the five EU countries that use the most animals overall: France, Germany, the United Kingdom (UK), Italy, and Belgium. These five countries, when taken together, account on average for 80% of the total animal use in toxicology by the EU, and each has used more than 200,000 animals for toxicity testing in at least one reporting year. Moreover, we normalized each country's numbers, setting the total animal use for each country during its first reporting year as a baseline (100%), in order to more easily compare trends across countries. Thus, we can see the relative change in animal use in each country since 1999 (2002 for Germany).

We found that there is little consistency in the levels of animal use in toxicology across countries over the decade in question (Figure 1.6). Italy's numbers consistently (and dramatically) dropped, Belgium's numbers steadily rose, Germany's numbers remained fairly steady, and France and the UK showed more complicated patterns. France's numbers increased initially and then ended with a substantial decline, whereas animal use in the UK declined for many years but ended with a substantial increase from 2005 to 2008.

Looking across these five countries as a whole, however, the overall trend is one of modest decline, with the number of animals used for toxicity testing in 2008 totaling 87% of those used in 2002. This recent decline could be evidence that, notwithstanding the influence of various factors tending to drive up animal numbers in toxicology (e.g., REACH), the 3Rs are actually bringing animal numbers down. This remains speculative in the absence of further evidence.

Yet even in the absence of such information, it is clear that much remains to be done in applying the 3Rs in toxicology. Although no precise estimates are available, animal use in toxicology worldwide is still counted in the millions of animals. In light of the unfulfilled potential of 3Rs activity within toxicology, we next address some of the major challenges ahead.

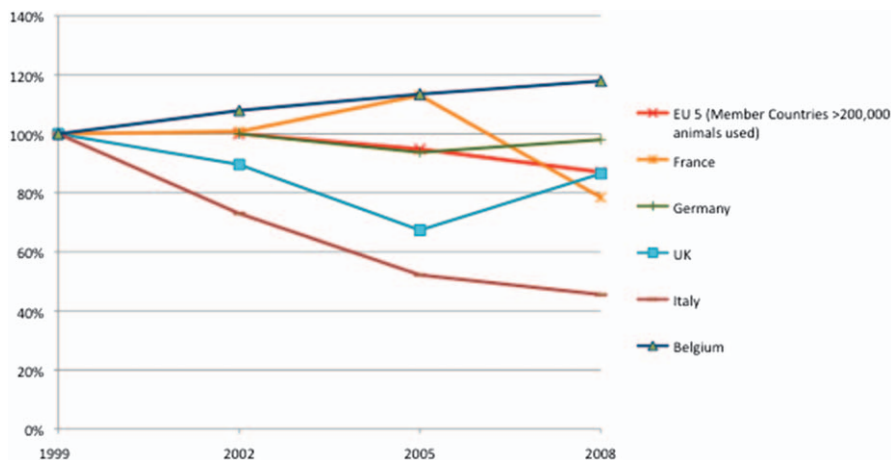


Figure 1.6 Animal use in toxicity testing in the European Union (EU) (1999–2008), based on European Commission reports to Parliament and working documents on the Statistics on the Number of Animals Used for Experimental and Other Scientific Purposes in the Member States of the European Union. Data shown only for those member countries using >200,000 animals in toxicity testing in a given year (France, Germany, United Kingdom, Italy, Belgium), which collectively account for approximately 80% of the total number of animals used in toxicity testing in the EU. No data are shown for Germany in 1999 because data were compiled differently that year, and therefore no data are shown for EU5 (the combined animal use from the 5 countries listed) for 1999. For ease of comparison, data are normalized such that animal use for the first reporting year is set to 100% for each country, and animal use in other years is expressed as a percentage of that year. Data tallied from boxes 2.4 (production and quality control of products and devices for human medicine and dentistry), 2.5 (production and quality control of products and devices for veterinary medicine), and 2.6 (toxicological and other safety evaluations, including safety evaluations of products and devices for human medicine and dentistry and for veterinary medicine) of the reports.

1.6 Remaining Challenges

In the decades following publication of Russell and Burch's *The Principles of Humane Experimental Technique* in 1959, the emerging 3Rs community devoted much of its energy to the field of toxicology. An infrastructure for making progress was slowly and steadily developed. This included alternatives-based organizations and centers, journals, websites, laws, conferences, and other activity. The 3Rs community also spearheaded the development of the scientific standards (e.g., validation) and worked with fellow toxicologists and others to improve the techniques (e.g., tissue culture) that facilitated progress. The result has been a progressive chipping away at traditional animal-based methods.

The publication of the NRC report on *Toxicity Testing in the 21st Century* in 2007 created a new point of reference for the 3Rs community, suggesting a new approach to replacing animal use in toxicology. Within the mainstream

toxicology and public health communities, the NRC report engendered considerable enthusiasm for modernizing toxicology. Leading federal scientists began predicting a paradigm shift from *in vivo*- to *in vitro*-based methods (see Section 1.1). Many in the alternatives community have been seeking to facilitate this long-term effort.^{39,47} As pathway-based approaches are further elaborated, they can be incorporated into ongoing 3Rs efforts.

However, implementing pathway-based approaches to predictive toxicology raises a host of formidable challenges.⁴⁸ These include developing fit-for-purpose assays to monitor pathway perturbations, distinguishing adverse responses in these assays from homeostatic responses,⁴⁹ accounting for metabolism of the parent compound, and shifting to a safety-based risk assessment paradigm, rather than one based on hazard.¹⁰

21st century toxicology efforts have the long-term goal of providing a new paradigm for safety assessment; however, for the time being, most of these efforts are being harnessed to predict the outcomes of traditional animal testing. Consequently, the first applications are supplementing rather than supplanting the current paradigm. We need to accelerate the transition to the new paradigm, based on human biology.⁴⁷

20th century validation processes will need to be adapted to 21st century toxicology.⁵⁰ There are many reasons why the existing validation processes should not be deployed as is. First, pathway-based methods are not intended as one-to-one replacements of animal-based tests; multiple pathway-based assays, perhaps numbering in the hundreds, will be used to make predictions about individual chemicals. Second, pathway-based methods ultimately will be called on to mimic human biology, rather than to predict the results of animal testing – the standard assurance of relevance. Third, according to the NRC framework, pathway-based assays will be used to predict regions of safe exposures, rather than predict specific toxicities. And fourth, the science and technology of 21st century toxicology are changing too rapidly for an evaluation process that takes a year or more to complete.

Most of the existing validation *principles* may carry over and transcend a paradigm shift in testing, but the prevailing validation *procedures* will need to be translated to accommodate these new realities. Validation procedures must also somehow be speeded up to accommodate the new pace of change and be flexible enough to accommodate an ideal of continual improvement in testing methods. Some rethinking of validation has begun to occur in this context.^{50–52}

And finally, perhaps the biggest real-world challenge to further progress on the 3Rs in toxicology is tackling systemic and chronic toxicity testing. Some large-scale programs in Europe have been seeking to address elements of this challenge, such as REPROTECT (reproductive toxicity)⁵³ and SEURAT-1 (repeat dose systemic toxicity testing),⁵⁴ with coordination and guidance provided by the AXLR8 program.^f In the U.S., high-throughput testing is being used to identify biological signatures of chronic, systemic endpoints, such as in developmental and reproductive toxicity.^{55,56} Clearly, integrated testing

^f<http://axlr8.eu>

strategies involving multiple types of testing and approaches (e.g., pathway-based testing, high-throughput testing, toxicogenomics, organ-on-a-chip platforms, virtual organs) will play a role. Also critical will be a realistic assessment of the limitations (as well as the strengths) of the current animal-based assays.²⁵

1.7 Conclusions

Progress on the 3Rs has been driven by a dual concern for animal welfare and scientific advancement. Much of this progress to date, especially in toxicology, can be attributed to the efforts of those who would identify themselves as part of the 3Rs or alternatives community and can be traced back in time to the pioneering efforts of William Russell and Rex Burch. What is especially exciting about the current era is that the 3Rs community is now working in parallel with a vanguard in the toxicology community seeking to usher in new approaches. Time will tell whether we are at the threshold of alternative approaches becoming the mainstream of the new toxicology.^{34,39}

Appendix A: Citation Search Strategies

We searched Web of Science, BIOSIS, and SCOPUS databases for citations to Russell and Burch's *The Principles of Humane Experimental Technique*, whether in its original edition⁴ or its 1992 reprint,³⁰ and the NRC's *Toxicity Testing in the 21st Century: A Vision and a Strategy*⁹ from the year of their respective publication to 2011, the latest year for which complete records were available. In addition, we searched SciSearch and Google Scholar for citations to *The Principles* up to and including the year 1999 to help compensate for the likely under-representation of papers and book citations during these years in the main databases.

For Web of Science, BIOSIS, SCOPUS, and SciSearch, we limited our results to publications related to toxicity testing by using the appropriate database subject/research area limits. In some cases, databases grouped toxicology and pharmacology together as one subject area. In others, the subject areas were distinct and pharmacology was included only if it was defined to include topics of relevance to toxicity testing. For the Google Scholar search, no subject area limits were available, so we curated our results to remove any publications not clearly related to toxicology.

We adopted a search strategy that would be robust enough to capture variations in how the author names and titles were entered into the databases, including misspellings. Searches were conducted October/November 2012.

Databases

1 Web of Science

According to its website, "Web of Science[®] provides access to citation databases with multidisciplinary content including Open Access journals and over

150,000 conference proceedings. It includes current and retrospective coverage in the sciences, social sciences, arts, and humanities, with coverage to 1900.”^g The relevant subject filters were “Toxicology” and “Pharmacology & Pharmacy.” “Toxicology” covers “resources that focus on the identification, biochemistry, and effects of harmful substances, including the side effects of drugs, in animals, humans, and the environment.” “Pharmacology & Pharmacy” covers “resources on the discovery and testing of bioactive substances, including animal research, clinical experience, delivery systems, and dispensing of drugs. This category also includes resources on the biochemistry, metabolism, and toxic or adverse effects of drugs.”^h

2 BIOSIS

According to its website, BIOSIS Citation Index “covers all major areas in the life sciences, with broad coverage in molecular and cell biology, pharmacology, endocrinology, genetics, neurosciences, infectious diseases, ecology and organismal biology. It provides access to over 22 million records from journals, books, reports, meetings, and U.S. patents dating 1926 or later.”ⁱ Subjects are defined in the same way as in Web of Science (above).

3 SCOPUS

According to SCOPUS, the database “is an abstract and citation database of peer-reviewed literature with tools that track, analyze and visualize research. Scopus includes over 20,500 titles from 5,000 publishers worldwide, 49 million records (78% with abstracts) and over 5.3 million conference papers.”^j The relevant subject filter in SCOPUS is “Pharmacology, Toxicology and Pharmaceutics” (no further description available).

4 SciSearch (*Accessed via DialogWeb*)

According to DialogWeb, SciSearch is “an international, multidisciplinary index to the literature of science, technology, biomedicine, and related disciplines produced by Thomson Scientific. SciSearch contains all of the records published in the Science Citation Index[®] (SCI[®]), plus additional records in engineering technology, physical sciences, agriculture, biology, environmental sciences, clinical medicine, and the life sciences. SciSearch indexes all significant items (articles, review papers, meeting abstracts, letters, editorials, book reviews, correction notices, etc.) from more than 6,100 international scientific and technical journals. Citation indexing allows for the searching of cited references.”^k

^ghttp://thomsonreuters.com/products_services/science/science_products/a-z/web_of_science/#tab1

^hhttp://ip-science.thomsonreuters.com/mjl/scope/scope_sci/

ⁱhttp://wokinfo.com/products_tools/specialized/bci/

^j<http://www.info.sciverse.com/scopus/about>

^k<http://library.dialog.com/bluesheets/html/bl0034.html>

5 Google Scholar

According to Google, “Google Scholar includes scholarly articles from a wide variety of sources in all fields of research, all languages, all countries, and over all time periods. Google Scholar searches across many disciplines and sources: articles, theses, books, abstracts and court opinions, from academic publishers, professional societies, online repositories, universities and other web sites. To be considered for inclusion, website content needs to meet two basic criteria: 1. Must include scholarly articles – journal papers, conference papers, technical reports, or their drafts, dissertations, pre-prints, post-prints, or abstracts . . . 2. Must show abstracts – websites must make either the full text of the articles or their complete author-written abstracts freely available and easy to see when users click on URLs in Google search results . . .”¹

Search Terms

1 Web of Science

We selected the “Web of Science” tab and then the “Cited Reference” tab. To retrieve citations to *The Principles*, we entered:

“Russel*, W*” in Cited Author AND
“Burch, R*” in Cited Author AND
“1959–2011” in Cited Year(s)

This produced 13 entries, of which we selected the 11 that were relevant to our search and clicked “Finish Search.” We refined the results by Research Area (on the left-hand side), selecting both Toxicology and Pharmacology/Pharmacy.

For citations to *Toxicity Testing in the 21st Century*, we located three entries, and the results were refined by Research Area to both Toxicology and Pharmacology/Pharmacy.

2 BIOSIS

We selected the “Select a Database” tab, then selected “BIOSIS Citation Index,” and from there selected the “Cited Reference” tab. We entered the same search terms as for Web of Science (above).

This produced 15 entries, of which we selected the 13 that were relevant to our search and clicked “Finish Search.” We refined the results by Research Area (on the left-hand side), selecting both Toxicology and Pharmacology/Pharmacy.

For citations to *Toxicity Testing in the 21st Century*, we located three entries, and the results were refined by Research Area to both Toxicology and Pharmacology/Pharmacy.

¹<http://scholar.google.com/intl/en-US/scholar/help.html#coverage>

3 SCOPUS

To find citations, we first needed to enter a publication known to cite the work in question, then locate the reference in that publication's entry. Next to the reference, there is text stating "Cited X times," with a link to the citations. Doing this, we were able to retrieve citations to both editions of *The Principles* and to the *Toxicity Testing in the 21st Century* report. SCOPUS does not have the same complication with variations of author name(s) and title as do Web of Science and BIOSIS.

4 SciSearch

We used SciSearch only for *The Principles* and entered the following search terms:

S1 CR = RUSSELL WMS, 1959?

S2 S1 AND PY = 197?:1999

S3 S2 AND (TOX? OR TEST?)

And:

S1 CR = RUSSELL W??, 1992?

S2 S1 AND PY = 1992–1999

S3 S2 AND (TOXIC? OR TEST?)

5 Google Scholar: <http://scholar.google.com>

We searched for "Principles of Humane Experimental Technique," and then selected the top result, which was for "The principles of humane experimental technique; WMS Russell, RL Burch, CW Hume – 1959 - altweb.jhsph.edu." Several other entries were listed for the book, but these had relatively few citations (generally less than 15 each), compared to more than 1400 citations for the entry we selected. As the database does not allow results to be restricted to certain research or subject areas, citations through 1999 were hand-curated to remove any not related to toxicology or pharmacology.

Limitations

Not all journal articles, books, or other documents will be indexed by the databases we used, and coverage is typically weaker prior to the early- to mid-1990s. In addition, some publications with relevance to toxicology may not be indexed under the Toxicology or Pharmacology research areas. While database indexers do their best to apply all relevant terms, it may have been particularly challenging to apply the Toxicology/Pharmacology label during the nascent

years of these fields, but also perhaps in more recent years as new specialties come to be identified as the primary focus.

To try to compensate for some of these factors, we included additional databases in our search for citations to *The Principles* prior to 2000. (There is likely to be greater overlap among the databases in more recent years.) While inclusion of these databases increased the absolute number of citations we were able to find, it did not significantly alter the general trend (data not shown).

Therefore, we expect that the total number of citations is likely higher than what we found, even with enhancement for the years prior to 2000, but the overall trend should remain valid. For *The Principles*, we see a low number of citations prior to 1980, a growth through the 1980s and 1990s, and a significant uptick starting in the mid-2000s. For *Toxicity Testing in the 21st Century*, we see an immediate uptake equaling, if not exceeding, reference to *The Principles*.

It is possible that the trend for *The Principles* largely mirrors the trajectory of toxicology publications in general. Advances in computer and internet technologies over the past one or two decades, for example, have had a tremendous impact on the number of and ease with which journals and papers can be published and indexed. As more papers get published, the number of papers citing *The Principles* has increased, but we do not know if the relative proportion of papers citing *The Principles* has decreased, increased, or remained steady. Notwithstanding this caveat, the trend in total number of citations could have looked very different, and our analysis shows a clear rise in influence of Russell and Burch's pioneering book during the last quarter of the 20th century and an enduring legacy into the 21st.

Appendix B: Literature Search Strategies

We conducted formal literature searches using Ovid Medline and Embase databases to identify a comprehensive, though not exhaustive, universe of 3Rs-related papers. Papers were considered to be related to the 3Rs if they used common 3Rs terminology or related synonyms, or if they were indexed as such by the databases. Several different search strings (queries) were devised to capture papers as comprehensively as possible while minimizing the number of irrelevant papers also captured. We did not, however, remove these "false positives" from our data set (of over 3000 records), which we estimated represented only 5–10% of our total.

We limited our results to those papers related to the field of toxicology, but did not include the field of pharmacology because these databases defined the field too broadly for our purposes. Instead, we included searches for vaccine safety and potency testing, which tended to fall in pharmacology rather than toxicology but are nonetheless related to toxicity testing.

We then categorized papers based on the occurrence of selected terms with 3Rs significance in the title, abstract, or key word fields of their database entries. Papers could be included in multiple categories if they contained more than one of the selected terms. While we selected a representative sample of important 3Rs-related approaches or concepts, such as humane endpoints and validation, no attempt was made to be exhaustive in the topics covered.

Searches were conducted during October 2012.

Databases

1 Ovid

According to Ovid, Ovid Medline is updated daily and “provides access to the latest bibliographic citations and author abstracts from more than 5,500 bio-medicine and life sciences journals in nearly 40 languages (60 languages for older journals). English abstracts are included in more than 80% of the records.”^m The “Toxicology Limit” was based on PubMed’s Toxicology subset limit.ⁿ

2 Embase

According to Embase, the database “covers international biomedical literature from 1947 to the present day. The database contains over 25 million indexed records and more than 7,600 currently indexed peer-reviewed journals. All MEDLINE records produced by the National Library of Medicine (NLM) are included, as well over 5 million records not covered on MEDLINE.”^o

Embase uses “Areas of Focus” filters, of which the relevant one for our purposes was “Toxicology and Drug Dependence.” According to Embase, this area “covers topics relating to toxic mechanisms and effects of both medicinal and non-medicinal substances. Included in this coverage are: Abuse of drugs, alcohol and organic solvents; Experimental pharmacology of addiction; Predictive toxicology. Coverage: records from 1983 to present.”^p The “Pharmacology and Pharmacy” area, as defined by Embase, dealt with topics largely outside toxicity testing.

Search Terms

Ovid

The following list of animal terms (“[list of animals]”) appeared in most of our searches:

(animal\$1 or rat or rats or mouse or mice or dog\$1 or cat\$1 or hamster\$ or gerbil\$ or “guinea pig\$1” or monkey\$1 or primate\$1 or rodent\$1 or rabbit\$1 or bird\$1 or fish\$2 or zebrafish or chicken\$1)

Indexing terms:

1) exp “Animal Use Alternatives”/ and (test or tests or testing or toxic\$.ab,ti,jw.

Alternatives to the use of animals:

2) ((alternative\$1 adj5 (“use” or “uses” or “using” or test or tests or testing)) and (“use” or “uses” or “using” or test or tests or testing) adj3 ([list of animals]))).mp.

^m<http://www.ovid.com/webapp/wcs/stores/servlet/ProductDisplay?storeId=13051&catalogId=13151&langId=-1&partNumber=Prod-901>

ⁿhttp://www.nlm.nih.gov/bsd/pubmed_subsets/tox_strategy.html

^o<http://www.embase.com/info/what-embase>

^p<http://www.embase.com/info/helpfiles/search-forms/advanced-limits/areas-of-focus>

- 3) ((alternative\$1 adj5 (“use” or “uses” or “using” or test or tests or testing)) and (“use” or “uses” or “using” or test or tests or testing) adj3 ([list of animals]))).mp. AND toxic\$.jw.

3Rs:

- 4) (3Rs or “Three Rs” or “Three R” or “3R principle” or “3R principles” or “3R approach” or “3R approaches” or “3R method” or “3R methods” or “3R concept” or “3R concepts” or “3R strategy” or “3R strategies” or (3R adj4 (alternative or alternatives))).mp.

Replace, reduce, and refine:

- 5) (Replac\$ and reduc\$ and refin\$).mp.

Animal Refinement:

- 6) (([list of animals]) adj5 refin\$.ti,ab. and (alternative or alternatives or test or testing).mp.

Minimize or eliminate pain or distress:

- 7) ([list of animals].ti,ab. and ((minim\$ or eliminat\$) adj4 (pain or distress)).ti,ab. and (alternative or alternatives or testing).mp.

Reduce, alleviate, or lessen pain or distress:

- 8) ([list of animals].ab,ti. and ((reduc\$ or alleviat\$ or less\$) adj4 (pain or distress)).ab,ti. and ((toxic\$ and chemical\$) or (toxic\$ adj3 test\$) or ((alternative or alternatives) and toxic\$)).ab,ti

Reduce, alleviate, lessen, minimize, eliminate, or decrease suffering:

- 9) ([list of animals].ab,ti. and ((reduc\$ or alleviat\$ or less\$ or minim\$ or eliminat\$ or decreas\$) adj4 suffer\$).ab,ti. and (alternative or alternatives or test or testing).ab,ti.

Animal replacement:

- 10) ((([list of animals]) adj5 replac\$) and (alternative or alternatives)).mp.
11) ([list of animals].ab,ti. and (replace and (alternative or alternatives)).mp.

Animal reduction:

Reduce the number of animals/Number of animals reduced:

- 12) (((reduc\$ or minim\$ or fewer) adj4 number\$) and (number\$ adj3 ([list of animals])) and (alternative or alternatives or testing or toxicity)).ti,ab.

Reduce the use of animals/Use of animals reduced/Animal use reduced:

- 13) (((reduc\$ or decreas\$ or minim\$ or eliminat\$ or fewer) adj6 (“use” or “uses” or “used”)) and (“use” or “uses” or “used”) adj6 ([list of animals])) and (([list of animals]) adj6 (reduc\$ or decreas\$ or minim\$ or eliminat\$ or fewer)) and (alternative or alternatives or testing or toxicity)).ti,ab.

Reduce animal testing/Animal testing reduced:

- 14) (((reduc\$ or decreas\$ or minim\$ or eliminat\$) adj5 ([list of animals])) and ([list of animals]) adj1 (test or tests or testing)) and ((reduc\$ or decreas\$ or minim\$ or eliminat\$) adj4 (test or tests or testing)) and (alternative or alternatives or testing or toxicity)).ti,ab.

Refine and Reduce:

- 15) ((refin\$ adj6 reduc\$) and (alternative or alternatives or test\$ or toxic\$)).ti,ab.

Vaccine Safety and Potency Testing:

- 16) ((vaccine or vaccines) and (safety test\$ or potency test\$ or batch test\$ or quality control) and (alternative or alternatives) and (in vitro or in vivo or ([list of animals]))).ti,ab.
- 17) Combine 1-2 and 4-16 with OR.
- 18) Limit 17 to toxicology.
- 19) Combine 3 and 18 with OR.

Embase

The following list of animal terms (“[list of animals]”) appeared in most of our searches:

(animal OR animals OR rat OR rats OR mouse OR mice OR dog OR dogs OR cat OR cats OR hamster* OR gerbil* OR “guinea pig” OR “guinea pigs” OR monkey* OR primate* OR rodent OR rodents OR rabbit* OR bird* OR fish* OR zebrafish OR chicken*)

Indexing Terms:

- 1) “animal testing alternative”/exp AND (test OR tests OR testing OR toxic*):ab,ti

Alternatives to the use of animals:

- 2) ((Alternative or alternatives) NEAR/5 (use or uses or using or test or tests or testing)):ti,ab AND ((use or uses or using or test or tests or testing) NEAR/3 ([list of animals])):ti,ab AND [toxicology and drug dependence]/lim

3Rs:

- 3) (3Rs OR “Three Rs” OR “Three R” or “3R principle” or “3R principles” or “3R approach” or “3R approaches” or “3R method” or “3R methods” or “3R concept” or “3R concepts” or “3R strategy” or “3R strategies” or 3R NEAR/3 (alternative or alternatives)) AND [toxicology and drug dependence]/lim

Replace, reduce, and refine:

- 4) Replac* AND reduc* AND refin* AND [toxicology and drug dependence]/lim

Animal Refinement:

- 5) (([List of animals]) NEAR/5 refin*):ti,ab AND (alternative or alternatives or test or testing) AND [toxicology and drug dependence]/lim

Minimize or eliminate pain or distress:

- 6) ([List of Animals]):ti,ab,de AND ((minim* OR eliminat*) NEAR/4 (pain or distress)):ab,ti AND (alternative OR alternatives OR test OR testing) AND [toxicology and drug dependence]/lim

Reduce, alleviate, or lessen pain or distress:

- 7) ([List of Animals]):ti,ab,de AND ((reduc* OR alleviat* OR less*) NEAR/4 (pain or distress)):ab,ti AND ((toxic* AND chemical*) OR (toxic* NEAR/3 test*)) OR (alternative OR alternatives AND toxic*):ab,ti AND [toxicology and drug dependence]/lim

Reduce, alleviate, lessen, minimize, eliminate, or decrease suffering:

- 8) ([List of Animals]):ti,ab,de AND ((reduc* OR alleviat* OR less* OR minim* OR eliminat* OR decreas*) NEAR/4 suffer*):ab,ti AND (alternative OR alternatives OR test OR testing):ab,ti AND [toxicology and drug dependence]/lim

Animal Replacement:

- 9) (([List of animals] NEAR/5 replac*) AND (alternative or alternatives) AND [toxicology and drug dependence]/lim
- 10) ([List of animals]):ti,ab,de AND replace AND (alternative or alternatives) AND [toxicology and drug dependence]/lim

Animal Reduction:

Reduce the number of animals/Number of animals reduced:

- 11) ((reduc* OR minim* OR fewer) NEAR/4 number* AND number* NEAR/3 ([list of animals]):ti,ab AND (alternative or alternatives or testing or toxicity):ti,ab AND [toxicology and drug dependence]/lim

Reduce the use of animals/Use of animals reduced/Animal use reduced:

- 12) (((reduc* or decreas* or minim* or eliminat* or fewer) NEAR/6 (use or uses or used)) AND ((use or uses or used) NEAR/6 ([list of animals]) AND (([list of animals] NEAR/6 (reduc* or decreas* or minim* or eliminat* or fewer)) AND (alternative or alternatives or testing or toxicity)):ti,ab AND [toxicology and drug dependence]/lim

Reduce animal testing/Animal testing reduced:

- 13) ((reduc* or decreas* or minim* or eliminat*) NEAR/5 ([list of animals]):ti,ab AND (([list of animals] NEAR/1 (test or tests or testing)):ti,ab AND ((reduc* or decreas* or minim* or eliminat*) NEAR/4 (test or tests or testing)):ti,ab AND (alternative or alternatives or testing or toxicity):ti,ab AND [toxicology and drug dependence]/lim

Refine and Reduce:

- 14) (refin* NEAR/6 reduc*):ti,ab,de AND (alternative or alternatives or test* or toxic*) AND [toxicology and drug dependence]/lim

Vaccine Safety and Potency Testing:

- 15) ((vaccine OR vaccines) AND ((safety NEXT/1 test*) OR (potency NEXT/1 test*) OR (batch NEXT/1 test*) OR “quality control”) AND (alternative OR alternatives) AND (“in vitro” OR “in vivo” OR ([list of animals]))):ab,ti
- 16) Combine #1-#15 with OR.

Categorization

We parsed our universe of 3Rs-related papers into various categories to assess the frequency of occurrence of select key topics. We assigned a paper to any categories for which the associated term(s) appeared in the abstract, title, or key word fields of the paper’s database entry.

- Replacement: “replac”
- Reduction: “reduc”

- Refinement: “refin”
- In vitro/Cell culture/Tissue culture: “in vitro,” “cell culture” and/or “tissue culture”
- Validation: “validat”
- In silico/(Q)SAR: “in silico,” “SAR,” “structure activity relation,” and/or “structure-activity relation”
- (Integrated) testing strategy: “testing strateg”
- Humane endpoint: “humane endpoint”
- Enrichment: “enrichment”

Limitations

In addition to those limitations identified for the citation search for *The Principles* (Appendix A), which apply equally as well to the literature search for 3Rs papers, additional factors must be considered in interpreting these results.

Our search could only retrieve those papers employing our specific search terms and formulations. Some proportion of 3Rs-related papers, however, may use terminology that is less specific and therefore not easily identified by searches designed to minimize false positives. Even the main 3Rs terms “replace,” “reduce,” and “refine” are relatively non-specific, thereby increasing the difficulty of locating papers discussing these topics.

Relatedly, papers may address 3Rs concepts without using 3Rs-specific terminology or without being framed as such. The lack of uniform publication requirements to explicitly describe the 3Rs implications of a given work exacerbates this difficulty. The growing number of 3Rs-related papers may partly reflect the increasing penetration of 3Rs terminology, however, and this in itself would be an indicator of the growing influence and sophistication of 3Rs ideas.

Even if papers used the 3Rs terminology employed in our search, they may not have been identified if the search terms did not appear in the title, abstract, or key words. Also, many early papers are not indexed in the databases with their abstracts, so only their titles are amenable to searching. This contributes to our underestimate of 3Rs papers prior to the 1990s. Database indexing terms, such as Medline/Pubmed’s “animal testing alternative,” are also not entirely reliable, resulting in many alternatives papers not being indexed as such. In some cases, though, the term may be applied to papers that are not clearly discussing the 3Rs, contributing to our false positives.

With regard to categorizing papers based on the 3Rs concept they address (e.g., replacement, reduction, refinement, validation, etc.), our search does not retrieve papers addressing these concepts if they are not indexed as toxicology papers. Thus, work on topics like refinement, humane endpoints, and enrichment, which is more likely to be general to animal research rather than specific to toxicology, would be underrepresented in our search even though such work is of use to toxicologists.

Further, papers were categorized based simply on the occurrence of selected terms. It is possible that some papers may use a term but not be about that concept. For example, papers may mention “replacement, reduction, and

refinement” to introduce the concept of the 3Rs, but may actually only address one of the Rs. Such papers, however, would get categorized under each of the Rs.

Despite these limitations, many of which are inherent to database searching, our analyses provide useful insight into the penetrance and explicit use of 3Rs terminology and concepts in toxicology over time.

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