Toxicological Approaches to Complex Mixtures

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This paper reviews the role of toxicological studies in understanding the health effects of environmental exposures to mixtures. The approach taken is to review mixtures that have received the greatest emphasis from toxicology; major mixtures research programs; the toxicologist's view of mixtures and approaches to their study; and the complementary roles of toxicological, clinical, and epidemiological studies. Studies of tobacco smoke, engine exhaust, combustion products, and air pollutants comprise most of the past research on mixtures. Because of their great experimental control over subjects, exposures, and endpoints, toxicologists tend to consider a wider range of toxic interactions among mixture components and sequential exposures than is practical for human studies. The three fundamental experimental approaches used by toxicologists are integrative (studying the mixture as a whole), dissective (dissecting a mixture to determine causative constituents), and synthetic (studying interactions between agents in simple combinations). Toxicology provides information on potential hazards, mechanisms by which mixture constituents interact to cause effects, and exposure dose–effect relationships; but extrapolation from laboratory data to quantitative human health risks is problematic. Toxicological, clinical, and epidemiological approaches are complementary but are seldom coordinated. Fostering synergistic interactions among the disciplines in studying the risks from mixtures could be advantageous. — Environ Health Perspect 101(Suppl 4):155–165 (1993).

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Introduction

There is a considerable body of literature on the toxicology of mixtures; however, our understanding of the significance of exposures to compounds in mixtures, in contrast to single exposures, is sparse. To date, toxicology has remained primarily the science of individual poisons, even though people are rarely, if ever, affected by single agents in isolation from other agents that might influence risk. Toxicological studies of mixtures inherently are difficult, and the science of studying mixtures is not refined or codified highly. Understanding risks from simultaneous or sequential exposures to multiple agents, particularly at low levels of exposure, is the single greatest challenge to toxicologists today. This paper is perspective in nature and constitutes a summary review of the role of toxicology in understanding the health effects of human exposures to mixtures of toxic chemical and physical agents. As used here, the term toxicology refers to laboratory investigations that do not involve human subjects but involve *in vitro* and *in vivo* experimental systems encompassing molecules, cells, tissues, and laboratory animals. Although not emphasized in this review, analytical chemistry is often incorporated into toxicological studies of mixtures.

This review summarizes the background and current status of toxicological studies of mixtures but is not intended to be exhaustive. The purpose is to present the scope of efforts in mixture toxicology, the manner in which toxicologists approach the issue, and the role of toxicological studies in relation to epidemiological and clinical studies. A more detailed treatise on issues surrounding the study of mixtures was developed by the National Research Council (NRC) Committee on Methods for the *In Vivo* Toxicity Testing of Complex Mixtures and was published as the text *Complex Mixtures* (*1*). The reader is encouraged to refer to that text; the present review does not attempt to reiterate or abstract its contents but presents some additional perspectives.

The approach taken in this review is first to summarize past, major efforts in the toxicological study of mixtures, because this background provides several examples that are used later to illustrate different issues and research approaches. A latter portion of this review describes in general conceptual terms how toxicologists view and approach the problem of mixtures. In doing so, this section also describes the role of toxicology in our understanding of the health risks from mixtures and contrasts the strengths of toxicology with those of epidemiology and clinical studies. The last section briefly discusses approaches to toxicological studies of specific mixture-related problems.

Past Toxicological Research on Mixtures

One can gain a view of how toxicologists envision the problem of mixtures and how they have approached that problem experimentally by examining the major research efforts of the past. The following summary does not recount all past work but indicates the principal areas of focus on mixtures to date. Studies of specific mixtures representing major thrusts are described, followed by mention of integrated programs that have been funded to address the issue of mixtures more broadly.

Research on Specific Mixtures

Tobacco Smoke. Tobacco (almost entirely cigarette) smoke is a complex mixture long studied by toxicologists and remains one of the most important mixtures affecting human health today. Early studies were summarized in 1967 by Wynder and Hoffmann (*2*), and research through the mid-1980s was summarized in detail in a 1986 International Agency for Research on Cancer (IARC) monograph (*3*). Research on cigarette smoke continues at present, with recent efforts directed toward comparisons of effects of conventional and alternate types of cigarettes (*4*), comparisons of alternate methods for animal exposures (*5*), and molecular mechanisms of smoke-induced mutagenesis (*6*). The full range of *in vitro* and *in vivo* toxicological assays has been used to study tobacco smoke, including aerosol characterization, analytical chemistry, dosimetry, metabolism, mutagenesis, teratogenesis, lung defenses, physiology, and long-term and acute inhalation bioassays. Nearly all past research on tobacco smoke was done using mainstream smoke; relatively little information has been published on sidestream or environmental tobacco smoke (ETS).

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It is instructional to consider the history of cigarette smoke toxicology in view of the present thrust to examine the health effects of mixtures, particularly ETS. That history will not be recounted in detail; suffice it to state that despite the hundreds of studies over five decades and the clear epidemiological signal for a range of adverse effects, the specific components of smoke causing the effects, the precise mechanism of the effects, and the reason for marked individual differences in susceptibility to the effects remain only partially understood. Toxicology has shown that cigarette smoke is cytotoxic, mutagenic, and carcinogenic, and that chronic exposure can cause chronic respiratory disease and impairment of lung defenses in animals. However, most of this information also is evident from observations of humans. The fact that toxicology has refined our understanding of the toxicity of tobacco smoke but has not yet resolved many key issues suggests caution against undue optimism for the study of ETS and other mixtures.

Toxicology has been hampered particularly by the lack of a good animal model for the pulmonary carcinogenicity of cigarette smoke as it is observed in man. Repeated heavy inhalation exposures of substantial numbers of subjects for the majority of a life span have been attempted only with rodents. It has been speculated that the failure of these studies to clearly demonstrate lung cancer induction resulted from the stressful, labor intensive, nose-only exposure methods used for exposures, which coupled with the avoidance responses of rodents exposed "puff-by-puff" have resulted in inadequate dosing. Indeed, compared to human heavy smokers, this is a somewhat unique example of underdosing in animal toxicological studies. In addition, studies to date have lacked statistical power by including small numbers of subjects living sufficiently long for cancer to be expressed. There is a current effort to use whole-body exposures in an attempt to overcome these difficulties (*5*), but it remains to be seen if the approach will succeed. Whole-body exposures also are being used in newly initiated studies of ETS sponsored by the Center for Indoor Air Research. Although *in vitro* and short-term animal studies suggest that nitrosoamino compounds may play a substantial role in the carcinogenicity of cigarette smoke, the relative contributions of the many potential carcinogens in smoke to pulmonary carcinogenicity of inhaled smoke remain unknown.

Combustion Products. There is a body of literature, summarized in the NRC book (*1*), on the acute effects of the products of flame combustion or heat pyrolysis of wood, plastics, and other materials. Studies, as exemplified by those of Alarie and Anderson (*7*), typically have used single, short exposures and have characterized effects by mortality, respiratory responses, and respiratory tract histopathology. These studies have been empirical and generally have treated each exposure atmosphere as a whole. Little information has been generated on chronic or repeated exposures, the mechanisms of response, or the components of the exposure atmospheres responsible for the effects.

Engine Exhaust. There is a body of literature (although perhaps surprisingly small) on the toxicology of gasoline engine exhaust. The earliest major studies were those begun in the early 1960s by the U.S. Public Health Service in Cincinnati starting with short- and long-term exposures of rodents (*8*) and followed in 1965 by the initiation of long-term exposures of dogs (*9*). The dog studies also included groups exposed to raw, irradiated exhaust and mixtures of sulfur dioxide and sulfuric acid. Mild changes in respiratory function were observed in physiological parameters during the 5-year exposures (*9*), and both physiological and histopathological changes indicative of chronic, terminal airway and centriacinar disease were observed after the exposures (*10,11*). This study is unique in that it is the only substantive long-term study of dogs exposed to inhaled nonradioactive toxicants, and it demonstrated the persistence of exhaust-induced lesions at 3 years after exposures ended.

More recently, studies at two laboratories of rats and hamsters exposed chronically to diesel engine exhaust also included groups exposed to gasoline engine exhaust. Neither laboratory published the results from gasoline exhaust exposures in detail. Results from a study of mice, hamsters, and rats exposed for 2 years to gasoline exhaust at the Fraunhofer Institute were presented in part in 1985 at an annual meeting of the American Association for Aerosol Research. Results from a study of rats and hamsters exposed for 2 years to gasoline exhaust at the Battelle–Geneva laboratory were described partially in two publications (*12,13*). The detailed results were submitted as a final report to the sponsors (Committee of Common Market Automobile Constructors [CCMC], a consortium of European automobile manufacturers), but the report has not been made public. Both the Fraunhofer and Battelle studies produced subtle physiological changes but little evidence of chronic lung disease and no carcinogenesis resulting from gasoline engine exhaust exposure. In another study at the Fraunhofer Institute (*14*), rats were treated with dipentylnitrosamine to induce a high background incidence of respiratory tract tumors and also were exposed chronically to gasoline exhaust. Exhaust exposure reduced the nitrosamine-induced lung tumor incidence but increased the incidence of nasal tumors.

Diesel exhaust is undoubtedly the complex mixture receiving the most intense toxicological study, surpassing even tobacco smoke in the number of funding sources and research institutions committing major efforts to the evaluation of potential health effects of occupational and environmental exposures (*15–17*). With major efforts beginning in the late 1970s, these studies continue at present in the United States and abroad. The first major commitment was that of the U.S. Environmental Protection Agency (EPA), which launched large-scale, multifaceted studies during the late 1970s based on the finding that diesel soot-associated organic compounds were mutagenic and the prediction that the use of diesel engines in the United States automobile fleet would increase to bolster fuel economy. This was followed by the initiation of research by automobile manufacturers (General Motors, Ford, Volkswagen, and a consortium of European automobile manufacturers) and government agencies (Department of Energy [DOE] and National Institute of Occupational Safty and Health [NIOSH] in the United States, the Japan Automobile Research Institute, and the German environmental agency), numerous university laboratories, and the Health Effects Institute. This research is probably the best example of an approach combining dose–effect studies of a "real life" complex mixture with dissecting studies to determine the most hazardous components of the mixture.

The studies of diesel exhaust have encompassed nearly the entire range of toxicological, epidemiological, and risk assessment approaches (*17*). The composition of exhaust from different engines and under different operating conditions has been analyzed extensively. Animals have been exposed acutely and chronically to whole exhaust and to filtered exhaust gases and vapors. *In vitro* mutagenicity and cytotoxicity assays have been used in the biodirected analytical chemistry of soot-associated organic compounds. Animals and cells have been exposed to individual compounds found in exhaust and to representative compounds attached to model carrier particles simulating diesel soot. Health effects have been evaluated at the molecular, cellular, tissue, organ, animal, and human levels. The effects of engine type, operating conditions,

and exhaust clean-up devices on the toxicity of emissions have been studied. Risk factors derived from animal studies and epidemiology have been compared.

It is useful to reflect on the extent of our understanding of potential health effects that has developed from over a decade of research on diesel exhaust. We know that inhaled diesel exhaust is toxic and has a potential for causing cancer; however, this was known in general terms before recent efforts began. We now know with great certainty that chronic inhalation exposure of rats to high concentrations causes lung cancer in a dose-related manner and that this effect is associated with the carbonaceous core of the soot fraction of exhaust. We now know with some confidence the approximate upper bound of risk for lung cancer among exposed humans; our confidence is bolstered because we have substantive epidemiological information to complement the large base of toxicological data. We do not yet know, however, if the dose–response data from rats can be used with much accuracy to estimate unit risks for humans. This is because the mechanism of cancer induction in rats remains uncertain, and it is possible that the mechanism might not be relevant to human risk. We know that soot exposure increases DNA adduct levels in the lung cells of animals, but we do not know if this is related to mutagenic, soot-associated compounds. There is good evidence that nitroaromatic compounds in soot extract are responsible for much of the bacterial mutagenicity of the extract, but we do not know the relative contributions of these and other carcinogenic compounds to the pulmonary carcinogenicity observed in animals and thought to occur in man.

It is worth noting that the diesel exhaust research effort also has included the only major studies of the toxicology of combined exposures to mineral dusts and exhaust, perhaps the most complex mixtures studied to date. The NIOSH study (*18*) included rats exposed for 24 months to atmospheres containing diesel exhaust at 2 mg soot/m^3, coal dust at the same concentration, and a combination of diesel exhaust and coal dust at 1 mg/m^3 each. Among the several health-effects endpoints evaluated, the effects of diesel exhaust and coal dust were similar, with coal dust being slightly less toxic. No synergistic interactions between the exposure materials were noted. The series of studies of diesel exhaust at the Inhalation Toxicology Research Institute included one (*19*) in which rats were exposed for their life span to diesel exhaust at 3.5 mg soot/m^3, to either raw or retorted oil shale dust at 5 mg/m^3, or to combinations of diesel exhaust and the shale dusts at total particle concentrations of 8.5 mg/m^3. Although the results have not been reported fully, the effects of diesel exhaust and shale dusts generally were less than additive for delay of particle clearance; additive for respiratory function impairment; and greater than additive for lung collagen, airway fluid indicators of inflammation, and lung tumors.

Air Pollution. Outdoor air pollutants present the classic problem of understanding the toxicology of mixtures of agents that are physically and chemically complex. Most research on air pollutants has focused on individual chemicals and compounds rather than on mixtures, although the engine exhaust studies described above certainly address a complex component of outdoor air pollution. There has been little research on the toxicology of actual ambient air pollutant mixtures largely because of the complexity and variability of outdoor air contaminants. One of the few examples is Bils' 1966 study (*20*) in which mice of different ages were exposed to ambient Los Angeles air during pollution alerts.

One step removed from studying actual ambient air pollution is the study of synthetic pollutant mixtures generated by reacting selected compounds. An early example of this approach is Bils' 1967 study (*21*) in which he exposed mice to synthetic smog produced by reacting propylene, nitric oxide, and carbon monoxide under ultraviolet radiation. A more recent example is the effort at the University of California, Irvine Air Pollution Health Effects Laboratory, involving exposures of animals at rest and during exercise to mixtures generated by reacting various combinations of ozone, sulfur dioxide, nitrogen dioxide, sulfuric acid, ammonium bisulfate, and ferric oxide (*22,23*).

The third approach used to study air pollution mixtures is the study of the effects of two representative air pollutant constituents administered alone and in combination. The most extensive database is comprised of studies addressing interactions between oxidant gases and acid aerosols. As reviewed in 1990 by Last (*24*), synergy was reported between the oxidants ozone and nitrogen dioxide and between acid aerosols in collagen synthesis and inflammation in rats exposed to the agents singly or in combination. Kleinman et al. (*22*) exposed rats to combinations of ozone and/or nitrogen dioxide with acid sulfates. Those investigators observed synergy between ozone and sulfuric acid in the development of lung lesions. Schlesinger et al. (*25,26*) studied the effects of nitrogen dioxide and sulfuric acid aerosol, alone and in combination, on particle clearance mechanisms. The effects of mixtures differed from those of the single agents, although a consistent pattern of synergy was not observed.

Major Programs Funded to Study Mixtures

DOE Complex Mixtures Program. During the mid-1970s, the Energy Research and Development Administration (ERDA), the predecessor of DOE, initiated a major research effort on the toxicology of complex chemical mixtures. This program was predicated on the emergence of alternate fossil fuel technologies that involved process streams, emissions, or products consisting of chemical mixtures. This was the first major, agency-sponsored, multilaboratory effort to understand and estimate the health risks of toxic mixtures. Continued by the DOE and administered by the Office of Health and Environmental Research (OHER) within the Office of Energy Research, the program reached a peak annual funding level of approximately $57 million in fiscal year 1981. The majority of this research was conducted at five DOE laboratories: Argonne National Laboratory, Inhalation Toxicology Research Institute, Los Alamos National Laboratory, Oak Ridge National Laboratory, and Pacific Northwest Laboratory (although researchers at several universities also were involved).

During the early 1980s, therefore, the emphasis of the DOE program shifted away from assessing technology-specific mixtures toward gaining a more fundamental knowledge of how complex chemical mixtures interact with people and the environment. The complex mixtures program diminished during the mid-1980s concurrent with the shift of OHER's health-effects research emphasis toward the more basic mechanisms of disease induction by energy-related materials and the emergence of the human genome program. The Complex Chemical Mixtures Program ceased to exist as a formal entity during the late 1980s, although OHER continues to sponsor some chemical toxicology research.

Many of our current approaches to studying mixtures have roots in the DOE program. It spurred advances in analytical chemistry and methods for sampling complex atmospheres and process streams. It fostered the development of bioassay-directed chemical analysis, which remains a mainstay of the field. It demonstrated the usefulness of modifying complex mixtures

to reduce toxicity (i.e., the reduction of the mutagenic potential of hydrocarbon mixtures by hydrogenation). The DOE program also fostered the development of air, aqueous, and solid media transport models for chemicals and mixtures of chemicals. It contributed substantial literature on the tissue, cellular, and molecular dosimetry of chemicals. The DOE program generated risk-assessment paradigms for dealing with complex exposure issues.

NIEHS Hazardous Chemical Mixtures Program. Beginning in approximately 1983, The National Institute of Environmental Health Sciences (NIEHS) and the NIEHS National Toxicology Program (NTP) began an initiative to study the toxicity of chemical mixtures, which continues at present (*27*). A major thrust began in 1985 to study mixtures of chemicals identified as groundwater contaminants (*28*). This program began as an interagency agreement with the Agency for Toxic Substances and Disease Registry (ATSDR) in response to the Comprehensive Environmental Response, Compensation and Liability (Superfund) Act.

This program took the approach of developing a standard mixture of compounds identified as groundwater contaminants and studying the toxicity of the mixture as a whole in a variety of test systems. Nineteen organic and six inorganic chemicals were selected from those identified in groundwater in the vicinity of hazardous waste disposal sites by two surveys sponsored by the EPA. The initial study involved rats and mice exposed for three months via drinking water to the mixture at concentrations ranging from 0.1 to 1000 times a baseline concentration of each chemical, which was the average concentration of each chemical near the waste sites. A variety of mortality, morbidity, histopathological, clinical chemistry, cytogenetic, and neurobehavioral endpoints were evaluated during exposures and up to 3 months after exposure. The number of chemicals and their proportional concentrations remained constant; there was no adding, subtracting, or manipulation of the ratios of individual chemicals to examine synergy or cause–effect relationships. Although its scope has broadened since these first studies, this program remains one of the best examples of an approach that uses synthetic mixtures containing large numbers of chemicals.

This program continued with studies funded from NIEHS/NTP and studies done in collaboration with the EPA's Health Effects Research Laboratory (HERL), and Brookhaven National Laboratory complemented the Superfund studies. In fiscal year1989, studies of pesticide and fertilizer mixtures and of the effect of chemical mixtures on bone marrow cell proliferation after irradiation were added to the program. Collaborative studies with EPA/HERL focused on neurobehavioral toxicity and hepatotoxicity. The fiscal year 1990 funding level for this effort was approximately $3.5 million. Publications resulting from the toxicological studies began to appear in 1989 (*29–31*), and several reports are in the publication process.

Research on Sequential Exposures

The issue of sequential exposures to single agents or mixtures is linked inextricably to the issue of simultaneous exposures to multiple agents as mixtures. Although sequential exposures have been studied, the present information base is smaller than that for mixtures. The most extensive example of research on sequential exposures is the study of initiation–promotion phenomena in carcinogenesis (*32*). Another example, closely related to concerns for environmental exposures to mixtures, is the work of Chameaud and French co-workers (*33*) on interactions between cigarette smoke and radon in causing lung cancer. Although numerous issues of interpretation remain, Chameaud and colleagues reported that exposure of rats to smoke before exposure to radon caused no more tumors than exposure to radon alone, but exposure to smoke after radon appeared to act synergistically to increase the lung tumor incidence.

The largest current singly funded program on sequential exposures is the DOE-sponsored research at the Inhalation Toxicology Research Institute on combined and sequential exposures to radiation and chemicals in the nuclear workplace. This program includes both *in vitro* and *in vivo* studies. An example of the former is the finding of Brooks et al. (*34*) that beryllium impairs the ability of cultured cells to repair DNA damage caused by radiation. Two long-term carcinogenesis studies are under way, one examining interactions between inhaled and retained plutonium dioxide particles and subsequent multiple exposures to whole-body x-irradiation (*35*), and the other examining interactions between inhaled plutonium dioxide and subsequent inhalation of beryllium (*36*). The latter study already has shown that small amounts of beryllium in the lung can retard the clearance of plutonium particles markedly, thus increasing the radiation dose. A third major study was initiated during 1991 to examine interactions between single exposures to radionuclides and chronic inhalation of cigarette smoke.

As is true for studies of mixtures, toxicologists are typically more able than epidemiologists to view the issues of combined or sequential exposures with a real hope of dissecting interactions in a definitive manner. Although precautions might be necessary, such as consideration of agents contained in feed, water, or culture medium, the exposures of animals or cells to multiple agents can be controlled in a manner not possible in studies of humans.

Toxicologists' Approaches to Studying Mixtures

This section describes, in general terms, the perspective of toxicologists toward the issue of mixtures. It describes the views that toxicologists likely have regarding the issues, the types of experimental designs typically used, and how their views and approaches might differ from those of epidemiologists and investigators conducting clinical studies (experimental exposures of humans).

Toxicologists have distinct advantages over epidemiologists in their greater ability to exert three general types of control over their studies of mixtures: *a*) control of the population (e.g., a selection of systems ranging from DNA to intact animals; a selection of species, strain, age, gender; and previous exposure history); *b*) control of exposures (e.g., precise knowledge of the type and concentrations of atmospheric constituents, and control of the timing of exposures); and *c*) control of endpoints (e.g., nearly unlimited selection of sampling time and frequency, use of invasive and destructive tests, consistency and completeness of health status evaluations). Toxicologists also have a much greater experimental latitude than those conducting clinical studies regarding the range of exposures and response endpoints. These advantages cause toxicologists to readily envision a broader range of experiments than can be envisioned by investigators studying humans.

Toxicologists also can link more directly exposures to effects than typically is possible in studies of humans. The fact that both the subject's preexposure history and exposures are known and controlled makes cause–effect linkage easier. In addition, the toxicologist often can determine that an exposure actually results in a dose to the tissue manifesting an effect. Their greater potential for linking exposure to effect and controlling extraneous influences leads toxicologists to consider studying a wider range of interactions (as described below) than is typical for investigators studying humans.

Of course, toxicologists have the major disadvantage that their data are not derived from humans but must be extrapolated to humans with varying (and sometimes huge) degrees of uncertainty. The results

of many toxicological studies are difficult to extrapolate directly to man because of the uncertainty that effects, resulting from high-level exposures, would occur at the lower levels of human exposure. This is largely a problem of not knowing the shape of the exposure–response relationship at low exposure levels. Moreover, it is often uncertain whether or not the mechanisms resulting in effects from high-level exposures are even operative at lower exposure levels. Building extrapolation bridges from *in vitro* test systems and animal responses to human responses continues to be a weakness of both toxicologists and investigators of humans. Despite the extrapolation problem, their experimental advantages engender in toxicologists an optimism about their ability to address the issue of mixtures.

Types of Toxic Interactions Envisioned by Toxicologists

Interactions among Components of Mixtures. Toxicologists generally use the term interactions when speaking of the combined effects of two or more agents. This use of the term is questionable; it might better be reserved for the physical–chemical interactions that occur between agents in a mixture, and the term combined effects used for responses. Regardless, interactions will be used in deference to common usage. The three most commonly considered types of interactions between two agents are additivity, synergism, and antagonism, with respect to each measurable effect of exposure. Different possible manifestations of these three types of interactions are described below and illustrated by equations. Two points must be made. First, the interactions described include both those for which examples exist and those for which examples are not known currently but must be considered as potentially operable in exposures to mixtures. The presentation is conceptual; no attempt is made to give specific examples. Second, there is presently no codified method used by toxicologists for expressing the following interactions in equation form. The symbology used herein is illustrative but not standardized and probably not optimal.

Additivity occurs when the combined effect of two or more agents (or components of mixtures) equals the sum of the individual effects. This can be represented by the following expression for the case in which two agents (1 and 2), each having effect A, have effect $A + A$ when administered together:

$$1 = A,\ 2 = A{:}1 + 2 = A + A. \quad [1]$$

Synergism is said to occur when the combined effect of two or more agents given together exceeds the sum of the effects of the agents given singly, as in

$$1 = A,\ 2 = A{:}1 + 2 = A + A + A. \quad [2]$$

In this case, a single effect, caused by both agents alone, is amplified or expressed in a more than additive manner when the two agents are given together.

An important caveat is necessary here. Toxicologists are likely to call the above response synergism even if only one dose of each agent is used in a three-group study (exposure groups 1, 2, and 1 + 2). However, the outcome, $A + A + A$, might not represent strictly synergism. The outcome simply might have reflected a nonlinear dose–response curve, particularly if agents 1 and 2 caused effect A by the same mechanism. That is, it also might be true that increasing the dose of either agent caused the apparently synergistic response, as in

$$1 = A{:}1 + 1 = A + A + A, \quad [3]$$

or

$$2 = A{:}2 + 2 = A + A + A. \quad [4]$$

This raises a critical point that is often overlooked by toxicologists—the need for understanding the dose–response relationship for both agents if interactions between them are to be studied. Studies using single doses of multiple agents are useful as an initial exploration of the potential for a nonadditive interaction; however, they are seldom definitive. Ideally, studies of interactions would include enough groups to examine the dose–response surface encompassing the entire range of exposure concentrations of concern for each agent involved in the combined exposures. This approach is not always possible, however. The dose–response caveat applies to the cases that follow; however, it will be assumed for simplicity that the dose–response relationship for each agent is known as linear through the range of interest.

A different case likely to be termed synergism by toxicologists is one in which an agent has an effect and another has none (or at least none measurable) but interacts with the first agent to yield an effect greater than that of the first agent given singly, as in

$$1 = A,\ 2 = 0{:}1 + 2 = A + A. \quad [5]$$

Yet another possible interaction that would be termed synergism could arise when the effects of two agents given singly differ, but the combined effects are greater than the single effect of one or both agents, as in

$$1 = A,\ 2 = B{:}1 + 2 = A + A,\ A + A + B,\ B + B,\ A + B + B, \quad [6]$$

or

$$A + A + B + B. \quad [7]$$

Antagonism occurs when the effect of two or more agents given together is less than the sum of the effects of the agents given singly, as in

$$1 = A,\ 2 = A{:}1 + 2 = A,\ a,\ 0, \quad [8]$$

in which the lowercase letter represents an effect qualitatively identical to but of a lesser magnitude than the effect represented by the uppercase letter (i.e., $a < A < A + A$), and 0 represents no effect. In parallel to the different cases of synergism shown above, antagonism also could occur if one agent has no effect when given alone, or if the two agents have different effects when given alone, as in

$$1 = A,\ 2 = 0{:}1 + 2 = a,\ or\ 0, \quad [9]$$

$$1 = A,\ 2 = B{:}1 + 2 = a + b,\ a,\ b,\ or\ 0. \quad [10]$$

Under the controlled conditions of toxicological studies, it is theoretically possible (and often practical) to dissect additional types of interactions in which the combined effects of multiple agents do not fit the above paradigms; that is, they are not simply equal to, greater than, or less than the sum of individual effects. Interactions between two agents could involve an additional, or third effect, not known to be caused by either of the agents given alone. Although this type of interaction has received little attention, there should be an awareness that these possibilities exist when studying mixtures. The epidemiologist has little potential for dissecting such an interaction from the complex interplay possible among the undocumented (and sometimes unknown) exposures that all of their subjects incur during life. Toxicologists have greater potential for discovering interactions because of their control of confounding factors, which gives confidence that unanticipated effects actually resulted from the experimental exposures and not from unknown exposures, and because of their ability to determine that exposures and effects are mechanistically linked and not just coincidental.

Cases of interactions involving an unexpected effect could arise either when the

two or more agents have different effects given alone or when one or more of the agents has no known effect when given alone. Examples of these interactions are illustrated by the following expressions:

$$1 = A, 2 = B{:}1 + 2 = C, A + C, B + C,$$

or

$$A + B + C, \quad [11]$$

$$1 = A, 2 = 0{:}1 + 2 = C$$

or

$$A + C. \quad [12]$$

An increased focus on mixtures is certain to bring with it an increased awareness of the potential for various complex interactions, particularly in view of our parallel increasing awareness of the complexity of biological mechanisms producing some effects. The developing awareness of the multistep process of carcinogenesis is an example. A mixture could contain agents affecting different steps (e.g., adduction of DNA and growth factor secretion) or alternate pathways contributing to the same steps (e.g., different growth factors). It will be left largely to the toxicologists to address this issue. As a simplistic example, it might be difficult for the epidemiologist to differentiate between the following cases, particularly if exposure to agent 2 was unsuspected:

$$1 = C; \quad [13]$$

$$1 = 0, 2 = B{:}1 + 2 = C; \quad [14]$$

and

$$1 = 0, 2 = 0{:}1 + 2 = C. \quad [15]$$

Interactions Resulting from Sequential Exposures. When considering toxic interactions among components of mixtures, it is important to remember that adverse effects also can result from interactions among agents encountered as a result of sequential exposures. Although this is not the same issue as simultaneous exposures to multiple agents in mixtures, it is related closely and of considerable importance. The potential for exposures to multiple agents, and thus for adverse responses due to toxic interactions among agents, is greater for sequential than for simultaneous exposures because the potential for different types of serial exposures is nearly unlimited. Both environmental and occupational exposures present numerous opportunities for the expression of combined effects due to sequential exposures. As a common example, interactions between cigarette smoking and occupational exposures to inhaled agents typically involve alternating rather than simultaneous exposures.

In epidemiological studies of the effects of mixtures, it must be remembered that effects that result from interactions among components of a mixture might alternately have resulted from, or been influenced by, previous exposures. Although the initial uptake of multiple agents might have been separated in time, the actual exposure of a critical cellular or molecular target site to either the parent agents or active metabolites still could have been simultaneous. One can envision the simultaneous exposure of a target site to two agents taken in at different times due to the retention time of the first agent or to differences in the rates of metabolism or excretion of the agents. When simultaneous exposure occurs, the effect manifested at the target site could be the same whether the subject was exposed to the agents as a mixture or sequentially. Toxicologists are expected to resolve these issues.

Toxicologists' Experimental Approaches to the Study of Mixtures

Test Systems. The toxicologist's toolbox contains a wide range of biological test systems from subcellular units to intact animals. Cell systems include cultured mammalian cells and bacteria, such as the Salmonella system for assessing bacterial mutagenicity (Ames test). Numerous species and strains of animals can be exposed to mixtures by all imaginable routes, although the majority of exposures are done by the oral, dermal, inhalation, intravascular, and intraperitoneal routes. There are few limitations on the range of health-effects end points that can be evaluated, although many of the more clinical types of assays, such as physiological measurements, are done more readily using the larger species. Most assays, such as histopathology, serum chemistry and urinalysis, can be applied to animals exactly as they are to humans; however, the assays requiring subject interaction, such as respiratory function or neurobehavioral function, are modified to differing degrees from those applied to man.

Toxicologists frequently incorporate analytical chemistry into their studies to characterize both exposure materials and biological samples. Extensive analytical capability also is used in concert with biological response systems in an interactive, decision-making mode to determine the constituent of a mixture responsible for an effect. The terms biodirected fractionation or bioassay-directed fractionation often are used for this approach. Biodirected fractionation has been used to determine the active agents in several mixtures, an early example being the use of the mouse skin carcinogenesis assay to determine the tumor-initiating fractions of cigarette smoke condensate (*37*). More recent examples are the use of short-term mutagenicity and cellular transformation assays to determine the mutagenic constituents of cigarette smoke (*38*) and diesel soot extract (*39*).

Basic Experimental Designs. There are three fundamental approaches to the toxicological study of mixtures. Although several terms are used for these approaches, the terms integrative, dissection, and synthetic will be used here. Toxicological studies of diesel exhaust will be used to illustrate the differences among these approaches and how they are interrelated.

The Integrative Approach. The integrative approach involves exposure of test systems to the intact mixture and conducting exposure-response studies to evaluate the nature and magnitude of the hazard associated with exposure. This is often the initial experimental approach to the study of mixtures of a generic nature, such as the real-life mixtures tobacco smoke and diesel exhaust, or representative mixtures, such as the 25-compound mixture of water contaminants studied in the NIEHS program. This is the type of toxicological study most related to epidemiology, and it is often used in clinical studies as well. The exposure regimen and biological end points used by toxicologists might be generalized and exploratory in nature if there is little advance knowledge of the hazard, or they might be narrowly targeted if particular hazards are recognized or suspected in advance. These studies are often observational or phenomenological in nature, but they also can be carefully targeted to test specific hypotheses. In addition, the observational studies are not superficial; they can provide a great deal of detailed information if properly designed. Good examples of integrative studies are the several long-term studies of the carcinogenicity of diesel exhaust [recently reviewed by Mauderly (*18*)]. Some of these studies also provided detailed information on a range of noncancer effects, such as that conducted at the Inhalation Toxicology Research Institute, which provided detailed information on dosimetry and particle clearance (*40*), inflammatory responses (*41*), effects on immune responses in lymph nodes (*42*), lung structure–respiratory function correlates (*43*), and adduction of lung DNA (*44*), as well as carcinogenesis (*45*).

The Dissection Approach. The dissection approach seeks to understand the contributions of individual constituents or families of constituents to the toxicity of the mixture. Studies of this type often follow

Table 1. Example of a matrix study of two agents (*A* and *B*).

		Doses of agent A		
		A_0	A_1	A_2
Doses of agent B	B_0	A_0, B_0	A_1, B_0	A_2, B_0
	B_1	A_0, B_1	A_1, B_1	A_2, B_1
	B_2	A_0, B_2	A_1, B_2	A_2, B_2

the demonstration of an adverse effect by integrative studies. The ultimate goal of dissection studies usually is to identify the active agent in order to *a*) determine the causal mechanism of the effect, *b*) develop more accurate risk estimates by using a dose term that is better focused than using the entire mixture as the dose term, or *c*) reduce exposures by reducing the amount of the agent in the mixture.

In the dissection approach, the mixture is separated into individual constituents or families of constituents, which are then tested for biological activity. Biodirected fractionation is a case of the dissection approach in which a mixture is separated progressively into fractions containing fewer and fewer constituents, and each fraction is tested in a biological response system in an iterative manner (*1*). The fractionation is biodirected in the sense that the biological response indicates which fraction to pursue in subsequent iterations. Although the implementation is not always easy, the approach is a conceptually straightforward method for identifying the cause of the biological activity.

In the case of diesel exhaust, studies employing the dissection approach actually preceded and led to the flurry of recent integrative studies. The dissection studies began with the finding of Kotin et al. in 1955 (*46*) that solvent extracts of diesel soot were carcinogenic to mouse skin. Two decades later, EPA investigators found that the extracts were mutagenic to bacteria (*47*). Biodirected fractionation was used extensively to locate the primary source of mutagenic activity in diesel exhaust in the aromatic hydrocarbon fraction of the soot-associated organic compounds (*48*) and resulted in a focusing of attention on the nitropolycyclic aromatic compounds (*49*). Biodirected fractionation does not always involve *in vitro* test systems and short-term assays. On a larger scale, but identical in philosophy, biodirected fractionation using long-term animal exposures was employed by the Fraunhofer Institute (*50*) to determine that the pulmonary carcinogenicity of diesel exhaust was associated with the soot fraction rather than the gas-vapor fraction. Similarly, long-term animal exposures were used recently at the Inhalation Toxicology Research Institute to determine that the organic fraction of diesel soot is not required for the effect (*51*).

The Synthetic Approach. In the synthetic approach, the toxicologist begins with simple, laboratory-synthesized mixtures of compounds or agents, and usually compares the effects of the mixture to the effects of the individual constituents. This approach is used to study interactions between specific agents, to study combined effects using simple systems, and to identify constituents responsible for effects by studying them in a sequential, additive manner. The goal is to gain an understanding of causal interactions among agents by studying a small number of constituents in a stepwise manner. These studies usually begin with two agents and sometimes use increasingly complex combinations of agents to work toward an understanding of the causative agents, or mechanisms, of the effects of the complete mixture to which humans are exposed.

The synthetic approach sometimes takes the form of a matrix study, in which the combined effects of two agents in a range of concentrations are explored in a series of experimental cells. An exposure–matrix approach to studying interactions between two agents, *A* and *B*, each at two concentrations, or doses, is shown in Table 1. In the matrix shown, the A_0, B_0 cell is the control group, and the top row and left column of cells represent graded treatments with single agents. The matrix, therefore, contains four cells in which interactions between the two agents can be observed. This approach has practical limitations; a very large, three-dimensional experimental matrix would be required to fully examine interactions among three agents. For this reason, the matrix approach is used typically to examine interactions between only two agents, as in the case cited earlier of the plutonium–beryllium study at the Inhalation Toxicology Research Institute (*36*). The matrix approach can be simplified by using single-dose levels or by studying only a few of the cells of a matrix involving more than two agents, as exemplified respectively by the studies of oxidants and aerosols by Last and Warren (*52*) and by Kleinman et al. (*22*).

Studies of diesel exhaust have involved different types of synthetic experiments. One type is represented by the work of Wolff et al., who examined the lung retention in rats of a model organic carcinogen, nitropyrene, inhaled either alone or adsorbed on a model particle, carbon black (*53*). Wolff et al. also compared effects on particle clearance and inflammation of multiple models of diesel exhaust constituents, nitropyrene, benzo[a]pyrene, sulfur dioxide, and particles, when administered alone and in combination (*54*). Quite a different type of synthetic experiment is represented by the work of Henderson et al. (*55*), who examined the mutagenicity of solvent extracts of diesel soot from an engine burning simple, laboratory-synthesized fuels containing single aromatic hydrocarbon compounds.

Examples of Toxicological Approaches to Specific Mixture Problems

Three current issues involving exposures to mixtures are used below as examples of problems and potential approaches involved in the toxicological study of mixtures. These examples were selected because they also are used in other papers discussing epidemiological and clinical study problems and approaches.

Environmental Tobacco Smoke and Nitrogen Dioxide: Effects on Lung Growth and Susceptibility to Infection

One difficulty in the study of ETS is defining and generating a representative exposure material. Environmental tobacco smoke is a mixture of exhaled mainstream smoke, sidestream smoke, and reaction products of the constituents of smoke and of smoke with other agents in the environment. Sidestream smoke, diluted and aged, is probably a useful simulant, and the simulation can be improved by the admixture of some mainstream smoke generated by a puffing device. Cigarette smoking devices practical for creating such a mixture are available commercially. Simulating ETS for toxicological studies is not practical when using actual exhaled smoke and a room environment. These factors may not influence the toxicity of ETS strongly; regardless, they are too variable to simulate well. Systems for exposing cells or animals to various dilutions of simulated ETS are readily fabricated, and a range of well-defined experimental cigarettes is available from the University of Kentucky Tobacco

Health Research Institute. Nitrogen dioxide (NO_2) is available in compressed gas cylinders or can be generated by vaporization of the N_2O_2 dimer (*56*).

Another difficulty in addressing this issue is the choice of an experimental model for lung development. Until more is known about the mechanisms controlling cell division and differentiation during growth, studies of lung development will continue to use developing animal lungs rather than cells in culture. A difficult choice is incurred by the differences among species in the maturity of the lung at birth and its postnatal development (*57*). The lungs of laboratory animals and man go through similar stages of development, however, and animals can be used successfully in developmental studies if care is taken that the stage of development, rather than age, is the basis of comparison. Most studies of perturbations of lung development have used rats, and this model is being used presently by Pinkerton and colleagues (unpublished data) at the University of California, Davis, to study the impact of ETS on lung development.

Based on the above considerations, a useful approach for studying the effects of ETS and/or NO_2 on lung growth would be to expose rats to various concentrations of these agents between 1 week and 5 months of age. These represent the ages at which rapid alveolarization of the lung begins and the number of alveoli is complete, respectively, and would approximate exposure of humans between birth and approximately 8 years of age. The primary end points would be morphometric, to examine structural effects, and biochemical, to examine connective tissue effects. Respiratory function measurements would be a useful adjunct, but might not be as sensitive as detailed morphometry. Exposure of rats to 10 ppm NO_2 from birth to 6 months of age was not shown to affect respiratory function evaluated by state-of-the-art functional assays (*58*).

The issue of susceptibility to infection can be addressed by both *in vivo* animal studies and *in vitro* cellular studies, such as the phagocytosis and killing of microorganisms by pulmonary macrophages. There is substantial literature on the effects of inhaled pollutants on the susceptibility of animals, particularly rodents, to infection with bacteria, but there have been fewer studies using viruses. The effects of oxidants in these models were reviewed in 1989 by Frampton and Roberts (*59*). Because viral infections of children are the concern, infectivity models using viruses would be more relevant than those using bacteria. Influenza viruses have been used most frequently as models for studying pulmonary defenses against viruses (*59,60*). Animal models have included rodents, rabbits, monkeys, and dogs.

Laboratory studies addressing the influence of NO_2 and ETS on viral infections in children should not just focus on susceptibility to infection by examining morbidity or mortality but also should address the ability to develop immune responses to relevant types of viruses. Furthermore, studies are needed that examine the interplay among susceptibility to viral infection; development and severity of acute parenchymal and airway inflammation; lung growth; and subsequent development of airway hyperresponsiveness, sensitivity to allergens, and asthma. This is clearly a tall order for the infectivity models of the past. An example of an approach showing promise for studying these interrelated phenomena is the canine adenovirus model being developed and used at the University of Arizona (*61*). This model has potential applicability for incorporating pollutant mixtures into studies of growth and airway responsiveness.

Acid Aerosols and Oxidants: Effect on Respiratory Morbidity

As described earlier, there have been toxicological studies of the combined effects of inhaled acids and oxidants. Schlesinger et al. at New York University (*25,26*) have examined interactions between NO_2 and acid sulfates in affecting alveolar macrophage function and mucociliary clearance of rabbits. Kleinman et al. (*22*) and Mautz et al. (*23*) at the University of California, Irvine, have studied the interactive effects of ozone and NO_2 with acid sulfates on respiration, lung surfactant, pulmonary histopathology, and cell proliferation in the respiratory tracts of rats. Last (*24*), at the University of California, Davis, has studied interactions between ozone and NO_2 and acid sulfates in changing connective tissue synthesis in airway tissues.

Although the particle clearance studies of Schlesinger et al. approach indirectly the issue of morbidity, the primary concern for human exposures to atmospheric acids and oxidants has been for morbidity in the form of airway responses and aggravation of asthma. These issues have not received much attention from toxicologists, partly because of the limitations of animal models for hyperreactive airways and responses during exercise. The guinea pig has been used for years as a model for airway constrictive responses to acute inhalation exposures to acids and oxidants. The airway responsiveness of the guinea pig appears more comparable to man than small laboratory animals. However, most studies have used gross changes in breathing pattern or breathing mechanics as the response end point. It is not clear how closely these end points are related to the responses measured in man by forced expiratory tests.

In a program at the University of Cincinnati, Leikauf and colleagues (unpublished data) are using the guinea pig model and both physiological and molecular end points to explore interactions between acid aerosols and ozone. This and similar approaches should provide information that is applicable to the issue of respiratory morbidity in humans, even though the experimental end points might be different from those applied to humans. Leikauf and colleagues are exploring the use of cDNA probes for mRNA expression of phospholipase A_2, endothelin, and fibronectin as markers of hyperreactivity, comparing these markers to results of physiological measures of airway constriction. A similar approach is being used for mucin and transforming growth factor beta (TGF-β), possible markers for hypersecretion. These approaches have potential for examining oxidant–acid interactions at exposure levels below those for which the physiological studies of the past have demonstrated effects.

Environmental Tobacco Smoke and Radon-Induced Carcinogenesis

There are no reports of toxicological studies of the potential carcinogenic interactions between ETS and radon, but the issue of tobacco smoking (mainstream smoke) and radon has been given some attention. As reviewed recently by Guilmette et al. (*62*), exposure to radon (progeny) has been linked to increased risk for lung tumors in rats in laboratories in the United States and France and in dogs in one study in the United States. Although attempts have been made to study interactions between radon exposure and smoking in animal studies, the results have an uncertain interpretive value. One significant problem is the lack of a good animal model for smoking-induced respiratory carcinogenesis, as described earlier. Without a reliable model for smoking-induced cancer, the ability to explore carcinogenic interactions between tobacco smoke and other agents is hampered.

Chameaud et al. (*33,63*) exposed rats to simulated mainstream cigarette smoke either before or after exposure to radon. The animals were placed in a dome into which mainstream smoke was drawn from several cigarettes that burned simultaneously,

rapidly, and continuously by drawing a vacuum through a cigarette manifold. The rats remained in a static atmosphere of the resulting high concentration (uncharacterized) of smoke for 15 min, and then the dome was flushed with clean air. Although the rats were exposed to smoke in ten 15-min sessions four times weekly for a year, the relevance of the exposure method and pattern to human smoking is uncertain. Regardless, these investigators found that, while exposure to smoke before exposure to radon had no effect on radon-induced tumor incidence, exposure to smoke after radon increased the tumor incidence 2- to 3-fold. Exposure to smoke alone did not induce significant incidences of lung tumors.

Cross et al. (*64*) exposed a small number of dogs via mask to smoke from 10 cigarettes/day, with smoke inhaled during every tenth breath for 4 to 5 years, and also exposed the dogs to radon. Although the study was not statistically robust, the incidence of lung tumors was lower in the smoke + radon group than in dogs exposed to radon alone.

The above results do little to resolve the issue of potential carcinogenic interactions between ETS and radon. The rat study suggested that smoke acted as a promoter of radon-induced carcinogenesis. If the current attempt to establish a model of tobacco smoke-induced carcinogenesis in rats using whole-body exposures (*5*) is successful, this model could be applied readily to studying interactions between smoking and radon. In the absence of an animal model demonstrating smoking-induced carcinogenesis, animal studies of ETS-radon interactions are of questionable value.

In vitro or a combination of *in vivo* and *in vitro* approaches might be used to determine if radon and tobacco smoke are synergistic in causing preneoplastic changes. Cultured cells can be exposed to radon, or the alpha particle irradiation from radon progeny can be simulated by irradiation from other sources. As an example, Thomassen et al. (*65*) used electroplated sources of plutonium-238 to irradiate primary cultures of tracheal epithelial cells and to compare the preneoplastic transformation (induction of growth variants) of tracheal cells by α-irradiation and direct-acting chemical carcinogens. This transformation assay could be used similarly to study interactions between α-irradiation and cigarette smoke or smoke condensate *in vitro*. An alternate approach would be to expose animals to radon and cigarette smoke and to determine preneoplastic transformation in primary cultures taken from the exposed animals. Neither of these approaches has been used to date.

Summary: The Role of Toxicology in the Study of Mixtures

Overall, toxicology provides a degree of experimental selection and control that has potential for providing more detailed information about hazards from mixtures than is possible with epidemiology or clinical studies. First, toxicology provides a means of evaluating whether or not exposure to a mixture poses a health hazard without relying on human experience. This is important for exploring risks from a new mixture or combination of exposures for which human experience has not yet been accumulated or identified. This also is important, however, for exploring risks from mixtures to which humans have been exposed but for which the effects cannot be strictly identified as having resulted from the particular exposure of concern.

Second, toxicology provides a means of determining the causal constituent among components of a mixture shown to cause an adverse effect. In some cases, this also might be possible in clinical studies but is never possible in epidemiological studies.

Third, toxicology provides a means of determining the mechanism by which an effect occurs. This includes determining interactions among mixture constituents that are responsible for the effect, the toxicokinetics resulting in the dose of the critical agent to the critical biological site, and the mechanism by which the adverse effect results from the critical exposure. In rare situations, the mechanism of response might be determined in clinical studies, but is beyond the capabilities of epidemiology.

Fourth, toxicology provides a means of exploring, in a precise, stepwise manner, the existence and nature of adverse effects resulting from exposures to multiple agents, ranging from simple combinations of two agents to chemically and physically complex mixtures. Again, this might be done to some extent in clinical studies but not by epidemiology.

On the other hand, using its nonhuman test systems, toxicology alone can seldom provide accurate estimates of human health risk from exposure to mixtures. The qualitative extrapolation from nonhuman test systems to man is often satisfactory and is strengthened by developing an understanding that the same basic biological mechanisms are operative in the test systems and humans. However, the quantitative extrapolation of exposure–effects data from nonhuman test systems to man is difficult typically and often impossible to accomplish with a high degree of confidence. Not only are humans a different species than those used in laboratory studies, but they also live in an environment that is much more complex than the experimental setting. For some nondestructive, readily measured end points, clinical studies can serve as an extrapolation bridge between toxicology and epidemiology.

Toxicology is an important predictive and dissective science that complements the observational science of epidemiology. It is just as impossible to fully duplicate the human environment in the laboratory as it is to exert experimental control over human exposures in the environment. There is a greater potential for using experimental exposures of humans as an extrapolation bridge between toxicology and epidemiology than has been exploited in the past. Animals certainly can be exposed in any pattern to any experimental atmosphere to which human subjects can be exposed, and there is good potential for simultaneous exposures of humans and animals in the same chambers. This constitutes an area for exploration.

Coordination of complementary research using toxicological, clinical study, and epidemiological approaches is a worthwhile and reasonably achievable goal that has received little attention. Research sponsors could give emphasis to fostering such coordination, and researchers in the three disciplines should make greater efforts to reach out toward each other. Many research sponsors fund work in two or all three disciplines, but there has been little effort to actually coordinate how issues are addressed by the disciplines. Few scientific societies or journals consciously integrate the disciplines, a noteworthy exception being the American Thoracic Society, whose meetings and publications serve as an intersection for the three areas of interest. For the most part, the three disciplines remain separate sciences with only superficial contacts among them.

Advantage could be gained by fostering collaborations among toxicology, clinical studies, and epidemiology in addressing health risks from environmental mixtures. The goal of this closer collaboration would be to identify the next step for each discipline, to identify opportunities for directly comparative studies among disciplines, and to identify points at which research could be handed off from one discipline to another. As an example, epidemiology (including environmental sampling) could provide toxicology with information on the composition of mixtures to which humans

are exposed, patterns of exposure, populations of concern, health outcomes of concern, and the level of effects observed (or observable). Clinical studies could provide information on short-term responses and dose–response relationships, biomarkers revealing short-term exposures and effects, and the likelihood of sensitive subpopulations. Toxicology, in turn, could provide feedback on the biological plausibility of the suspected exposure–response relationship, the potential for chronic disease resulting from repeated exposures, causal and predictive relationships between acute and chronic effects, finer definition of dose–response relationships, active constituents of mixtures, and the effect of exposure pattern. Coordinated clinical and toxicological studies could, in some cases, provide direct human–animal comparisons that bolster confidence in the relevance of animal responses and provide quantitative extrapolation bridges. Other forms of complementary cross-feed among the disciplines are envisioned; the preceding examples are only illustrative. In general, the goal appears worthwhile.

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