Chapter 9
Predicting Mixture Toxicity with Models of Additivity

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Abstract Researchers in numerous fields (e.g., pharmacology, entomology, toxicology, and epidemiology) have attempted to model the joint action of chemicals using simple formulas based only on knowledge of individual chemical toxicity or pharmacological effect (i.e., dose-response relationships). Collectively, these formulas are referred to as "additivity models," and they are based on concepts of additivity that include dose addition, independent action, integrated addition, and effect summation. In toxicology, additivity-based predictions are often compared to observed mixture data to assess the presence and magnitude of interactions (greater-than-additive or less-than-additive) among chemicals. These models can also be used to estimate the toxicity of a defined mixture for comparison to the observed toxicity of a related, but more complex, mixture. Alternatively, additivity models have been used to explore mechanisms of joint action. In general, the steps for investigating joint toxicity using additivity models include (1) deciding on which additivity model(s) to apply (e.g., dose addition, independent action, or

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both), (2) collecting dose-response data on individual chemicals and the mixture, (3) incorporating individual chemical data in an additivity model to generate predictions, and (4) comparing predicted to observed mixture responses. Many of the additivity models have a long and sometimes controversial history. This chapter provides background on several of the common additivity models, illustrates their application with examples, and discusses their advantages and limitations.

Keywords Joint action · Dose addition · Loewe additivity · Independent action · Response addition · Bliss independence · Integrated addition · Effect summation · Component-based approaches

9.1 Introduction

The toxicity of a mixture can be evaluated by treating the whole mixture as a single chemical or by investigating how components in the mixture contribute to the mixture's toxicity. Each approach has advantages and disadvantages. While whole mixture testing can offer a straightforward experimental design (e.g., one test article), the major impediment lies in relating the toxicity results to real-world exposures or to other mixtures of interest. Specifically, challenges include selecting the whole mixture for study, generating the test article in concentrated form in a volume sufficient for testing, and relating findings to the vast number of potentially similar whole mixtures. Since the actual toxicological evaluation of the whole mixture is the same as for single chemicals, the whole mixture approach will not be discussed in this chapter. Challenges in whole mixture research relating to test article selection and generation of test material can be found in the literature (Simmons et al. 2008; Pressman et al. 2010), and approaches for comparing across whole mixtures are discussed in a risk assessment context in Chap. 15. The focus of this chapter is on understanding mixture toxicity through evaluation of the components of the mixture. This chapter discusses approaches for using individual chemical data in simple mathematical models to predict mixture effects.

The first step in understanding the joint action of two or more chemicals (i.e., how they behave when they co-occur in an organism) is to formulate a testable null hypothesis based on what is known about the individual chemicals and to evaluate it empirically. In studying additivity, this hypothesis typically involves a key assumption that the chemicals will abide by a defined model of joint action (e.g., dose addition, independent action) and do not interact (pharmacokinetically or pharmacodynamically) to alter the response expected under the model. In other words, the null hypothesis of additivity describes the "no-interaction," "zero-interaction," or "baseline" situation.

Empirical evaluation of a null hypothesis of additivity requires data. For the additivity models considered here, the required data usually consist of dose-response data for each individual chemical in the mixture as well as dose-response data for one or more mixtures, each defined by specified proportions of the individual chemicals. Also, the null hypothesis of additivity is generally evaluated

for a single specified response; or, when several specific responses are of simultaneous interest, additivity is evaluated for each response separately.

Of course, the totality of an organism's responses to a tested mixture might include characteristics that are biologically distinct from those seen with any of the component chemicals. Such qualitatively different responses could be caused when the components combine chemically to form a new chemical that differs from all of the components, for example, the potent carcinogen *N*-nitrosomorpholine can be formed from coadministration of nitrogen dioxide and morpholine (Van Stee et al. 1995). Alternatively, qualitatively different responses can occur when a pharmacokinetic interaction among chemicals leads to the formation of a toxic metabolite that would not otherwise be formed by any of the components (Dobrev et al. 2002). Toxicodynamic interactions could also alter physiological behavior or histological structure so that new effects become prominent. Although the absence of dose-response data on these responses for the individual chemicals makes formal evaluation of the additivity null hypothesis problematic, the appearance of those novel responses only with exposure to the mixture and not to the individual chemicals would provide prima facie evidence of departure from additivity.

In general, evaluation of the null hypothesis of additivity proceeds by (1) using the individual component chemical dose-response data together with a specific additivity model to predict the expected response at various doses of the mixture and (2) comparing those predictions under additivity to observed dose-response data for the mixture. At least four kinds of outcomes are possible (Fig. 9.1):

- The responses of the tested mixture are close enough to predictions under additivity that they support the stated additivity hypothesis.
- The responses of the tested mixture occur at lower doses than predicted under additivity, contradict the stated additivity hypothesis, and indicate a potential greater-than-additive interaction among the component chemicals.

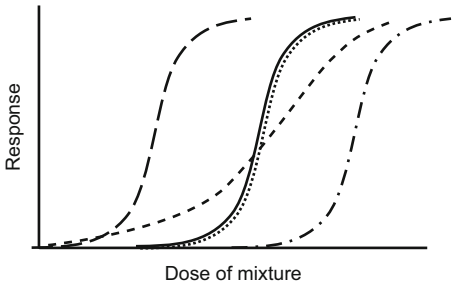

Fig. 9.1 Comparison of predicted and observed mixture responses. The solid black line represents predicted effects based on an assumption of additivity (a.k.a. "no-interaction" scenario). The dotted line represents observed mixture data that conform to additivity-based predictions. The long-dashed line represents mixture data that deviate from additivity, displaying greater-than-additive toxicity. The dash-dot line represents mixture data that deviate from additivity, displaying less-than-additive toxicity. Finally, the short-dashed line represents mixture data that deviate from additivity, displaying greater-than-additive toxicity in the low-dose range and less-than-additive toxicity in the high-dose range

- The responses of the tested mixture occur at higher doses than predicted under additivity, contradict the stated additivity hypothesis, and indicate a potential less-than-additive interaction among the component chemicals.
- The responses of the tested mixture occur at lower doses than predicted under additivity at some dose levels and higher doses than predicted under additivity at other dose levels, contradict the stated additivity hypothesis, and indicate potential interactions that are either greater-than-additive or less-than-additive, depending on the dose level.

Selection of an appropriate model is complicated by the fact that there are multiple additivity models, often with numerous versions, available in the mixture literature. Furthermore, it is important to note that deviation from an additivity prediction is not enough to confirm the presence of an interaction among mixture constituents. For example, an inappropriate additivity model may have been applied – a dose-addition model was used when the chemicals displayed independence. Conversely, consistency with model predictions does not necessarily rule out the presence of greater-than-additive or less-than-additive interactions. One can imagine, for example, that the presence of greater-than-additive interactions among some mixture components and less-than-additive interactions among others could counteract each other in some sense and result in responses that approximate additivity. Also, as determination of consistency with or deviation from a model is based on statistical comparisons between the predicted and observed responses, some real deviations from additivity may not be detected due to inadequacy of experimental design, including insufficient power (See Chap. 13), and some detected deviations may represent statistical false positives.

As suggested above, no definition of additivity is universally accepted. Instead, there are multiple definitions of additivity that can be separated into two basic categories: (1) additivity defined through formulas involving dose levels (e.g., dose additivity) and (2) additivity defined through formulas involving response levels (e.g., independent action and effect summation). There are also integrated-addition models, which combine the concepts of dose addition and independent action. For dose addition, multiple mathematical approaches are available for calculating the predicted response of the mixture from dose-response data on individual chemical constituents. This chapter will focus on the different concepts of additivity and the varied methods that have been used to predict mixture toxicity under an assumption of additivity. It will present the history, assumptions, current applications, and relevance of four general types of additivity (viz., dose addition, independent action, effect summation, and integrated addition).

Many factors must be considered when tested mixtures result in responses that deviate from those predicted under additivity. The first set of factors involves the series of decisions made in the modeling process. Typical examples include the quality of the individual chemical data, the dose-response modeling of the individual chemical data (Chap. 8), and the selection of the additivity model (e.g., was independent action applied, when a dose-addition model would have been more appropriate). The second group of factors relates to the simplicity of the models

versus the complexity of biology. The models described in this chapter are simple mathematical models, whereas the biological responses that are being evaluated often involve complex, interdependent signaling networks. The additivity models represent a reductionist approach that may provide different results depending on the model system and endpoints being evaluated. Additionally, chemicals can interact with biological systems in complex ways that are not captured by the models (e.g., a single chemical can activate multiple adverse outcome pathways).

Despite these complications, the models of additivity discussed in this chapter offer a starting place for understanding the joint action of chemicals. They can be used to address important issues in toxicology ranging from limiting the potential toxicity of combination therapies to informing the process of cumulative risk assessment. Sometimes, simple models of additivity will be adequate for describing mixture responses and will support continued application of the models for predicting the toxicological consequences of exposure to mixtures. Other times, information gained from applying different additivity models, such as patterns of deviation from the model(s), could inform the development of more sophisticated models of mixture toxicity that would better integrate component chemical dose-response data. Regardless, targeted mechanistic studies can be designed based on results from comparing observed mixture responses to predictions under additivity to further characterize and understand the joint action of chemical mixtures.

Displaying dose-response relationships for mixtures graphically can be useful for visualizing some key mixture concepts (Greco et al. 1995). For a single chemical, a dose-response relationship is typically depicted graphically as a curve with responses on the vertical axis and dose levels on the horizontal axis. With binary mixtures, graphs of the dose-response relationship that depict the responses and the dose levels of both chemicals are called response surfaces and require three dimensions, a vertical axis for response and two perpendicular "horizontal" axes for the dose levels (dose plane). (This idea generalizes mathematically to higher-order mixtures, but visualization is inconvenient at best.) Two-dimensional representations of features of response surfaces are often used. For binary mixtures with a fixed ratio of the two constituent chemicals, increasing doses of the mixture extend along a ray that lies in the dose plane and starts at the origin (zero dose of the mixture). A plane that is perpendicular to the dose plane and that contains the fixed-ratio ray cuts through the response surface; the set of points at the intersection of that perpendicular plane and the response surface is the dose-response curve for that fixed-ratio mixture. Treating the mixture as a single "chemical," the mixture's dose-response curve can be displayed in two dimensions just like any dose-response curve for a single chemical. One can also cut the dose-response surface with a plane parallel to the dose plane at any selected response level; this parallel plane also has the dose levels as its axes. The intersection of such a parallel plane with the dose-response surface is called an isobole: a line or curve that connects equi-effective doses on a graph whose axes are the dose levels of each component chemical. Thus, an isobole is always associated with a specified response level. A graph of isoboles is called an isobologram. See Greco et al. (1995) for a more thorough explanation and illustration of these ideas. As described later, isobolograms have been used widely for evaluating the additivity of binary mixtures.

9.2 Toxicological Similarity and Additivity Model Selection

The degree of similarity in toxicological responses to chemicals has generally served as the basis for selection of an appropriate additivity model, with dose additivity traditionally being applied to chemicals that elicit "similar" responses and independent action being applied to chemicals that elicit "dissimilar" responses. However, the terms "similar" and "dissimilar" have had varied interpretations. The spectrum of toxicological similarity among chemicals, along with examples of chemicals that correspond to each level, is presented in Fig. 9.2. As chemicals move down the spectrum of similarity, scientific support for applying the concept of dose addition to predict combined effects becomes more tenuous.

As noted in Fig. 9.2, chemicals with the highest degree of similarity share a common active metabolite (assuming that the parent compounds require metabolic activation). In this case, the metabolite responsible for initiating the cascade of responses is identical among chemicals. It follows that the sequence of biochemical steps resulting from the molecular initiating event is identical between all chemicals that share the active metabolite. Any difference in potency among chemicals could be attributed to differences in the rate and extent to which the chemicals are converted to the active metabolite and would be reflected in the individual chemical dose-response relationships.

The next level of similarity involves chemicals that elicit toxicity through a common molecular initiating event. There are many classes of chemicals that meet this criterion, for example, organophosphate pesticides that share inhibition of acetylcholinesterase as the molecular initiating event that leads to downstream adverse nervous system effects (Mileson et al. 1998). Toxicological similarity at the level of shared molecular initiating event has provided the conceptual basis for dose addition. The concept holds that chemicals displaying similarity at the molecular initiating event level act as dilutions or concentrations of each other, thus

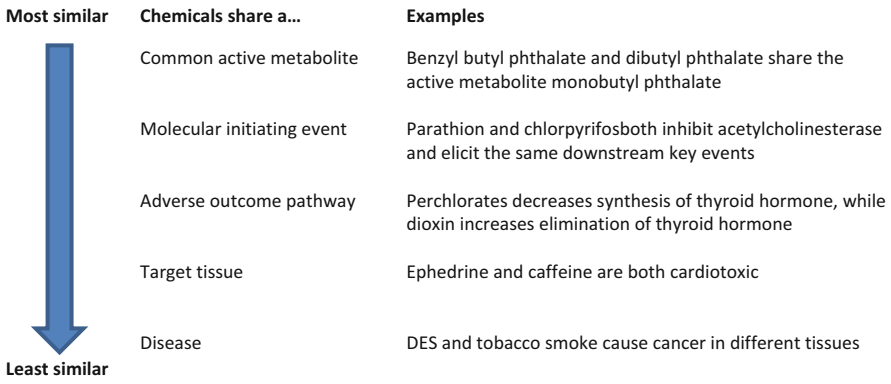

Fig. 9.2 Continuum of toxicological similarity

eliciting the same toxic effects by the same toxicological pathways, once dose is scaled for that "dilution" or potency factor (Cedergreen 2014; Hertzberg et al. 2013; Loewe and Muischnek 1926). The dilution concept, first described by Bliss (1939), can be empirically tested by determining whether the chemical components and the mixture share a common dose-response model, except for scaling the dose (Meadows et al. 2002), and in the most complete dilution concept, by determining whether the response variances depend only on the response mean and otherwise are the same across all chemical components and the mixture (Hertzberg et al. 2013).

It is at the level of adverse outcome pathway that the concepts of toxicological similarity and independence begin to blur. Chemicals can have different molecular initiating events that converge at any of a number of key events in their adverse outcome pathways (Chap. 7). Moving one step further, chemicals can act through different signaling pathways that intersect at the target tissue. Although a common target tissue among chemicals can be one of a number of factors used to build a "weight of evidence" case for toxicological similarity, there is continued debate on using shared target tissue alone as the basis for applying dose addition. Many chemicals have notably different adverse outcome pathways yet display toxicity at the same tissue (see Sect. 3.3). Determining how the degree of toxicological similarity of chemicals in a mixture relates to predicted mixture toxicity from available additivity models is an active area of research and has important implications for cumulative risk assessment (Chap. 14).

Regarding the concept of toxicological similarity, it is important to note that evidence for a concept is not the same as the lack of evidence against a concept. Evidence supporting independent action has often involved demonstration of differing adverse outcome pathways for the most important effects. Evidence supporting similar adverse outcome pathways has usually been interpreted as support for toxicological similarity in general and thus for the dose additivity model. For weakly studied mechanisms, a similar conclusion has been drawn based on the lack of evidence for multiple differing adverse outcome pathways, i.e., inability to reject toxicological similarity. Similarly shaped dose-response curves (generalized parallelism) have also been interpreted as indicating similar adverse outcome pathways, and thus dose addition, yet counterexamples exist (Hertzberg et al. 2013). Specifically, a "lack of a different slope cannot be taken as a proof for a similar mode of action" (Altenburger et al. 2005). The decision about sufficient toxicological similarity for inclusion in a similarity group is a judgment and thus requires involvement of experts in toxicology and data analysis.

Finally, evaluating the evidence for toxicological similarity necessarily involves consideration of data quality. Different evidence streams can support determination of similarity, including structural similarity among chemicals, consistency of toxicological profile, and similar findings in mechanistic studies used to build adverse outcome pathways. For risk assessment purposes, frameworks have been developed to make decisions on including chemicals into a toxicological similarity group (U.S. EPA 1999). Structural comparisons (e.g., quantitative structure activity relationships) have been used for a long time, but many counterexamples exist

regarding toxic effect, so structure alone is insufficient for defining a similarity group. For any chemical's toxicological profile, key elements include the target organ(s), adverse effects/apical outcome(s), and pharmacokinetic properties, including whether the parent or metabolite is the toxic form. The preferred information on a toxicological profile involves effects related to specific molecular targets (e.g., particular enzyme or other protein, hormone) or target tissues (e.g., thyroid, blood) as similar specific effects (e.g., receptor-mediated effects) are more likely to follow a common toxicity pathway than nonspecific toxicity (e.g., narcosis). Knowledge of the adverse outcome pathway and related pharmacokinetics is the best information for judging toxicological similarity; however, these data are often unavailable or incomplete.

In contrast to toxicological similarity and dose addition, most of the classical literature that presents component-based formulas for joint toxicity includes minimal biological justification for independent-action models. The most common justification is evidence of important differences in toxicological mode of action or mechanism; in some cases, that evidence is only of effects occurring in different organs. In a review comparing concentration-addition (CA) and independent-action (IA) formulas, the conclusion was that little biological clarity has been proposed: "The CA model has some theoretical underpinnings and some experimental data supporting its use for mixtures of chemicals with the same site of action. In contrast, IA is not as well grounded in theory, nor is it unambiguously supported by data" (Cedergreen et al. 2008).

The mechanistic or functional argument for independence should be based on data and concepts showing that an individual chemical's toxic function, dose-response relationship, and adverse outcome pathway are not altered by co-exposure to the other chemical(s) in the mixture. Because toxicological interactions are known to involve one or more key event(s) from exposure to apical (adverse) effect, an evaluation of independence should include as many of those steps as possible. For chemicals, the following processes have been identified as involved in toxicological interaction: contact, uptake, in vivo chemical reactions, absorption, distribution, excretion, metabolism, receptor site (antagonism), receptor function (antagonism), and DNA binding (Mumtaz and Hertzberg 1993; Mumtaz et al. 1993). Evidence only of a difference in toxicodynamics, i.e., in toxicological mode of action, thus provides fairly weak support for toxicological independence. For example, independence might not be plausible when adverse outcome pathways overlap for secondary effects: "commonality of biological/biochemical events that may not be part of the recognized toxic mode or mechanism of action of one mixture component can lead to unanticipated interactions and consequences" (Lambert and Lipscomb 2007). Such information is not commonly available. Usually independence and the independent-action formula are assumed when there is insufficient evidence to establish toxicological similarity.

Finally, the experimental endpoint that is selected in a mixture evaluation (i.e., measured response) can also influence the determination of similarity versus independence. The specificity of the endpoint measured could increase the likelihood of classifying chemicals as toxicologically independent. Consider the

difference between an in vitro assay measuring binding to a hormone receptor and a downstream functional measure of the system in which the hormone operates as a signaling molecule. In the case of the in vitro binding assay, there is a limited capacity for interactions between the chemicals and the system. Instead, there is a focus on a single, clearly defined mechanism. In contrast, the functional measure incorporates multiple mechanisms and potential avenues for interaction among chemicals and complex biological systems.

9.3 Dose Addition

The phrases "dose addition" and "concentration addition" represent the same concept, with "dose addition" used more frequently in mammalian toxicology and "concentration addition" commonly associated with ecotoxicology. For simplicity, the term "dose" will be used throughout this chapter. As indicated by its name, dose addition is a concept that defines or predicts the additive joint action of chemical mixtures by imposing a constraint on a weighted sum of the component-specific dose levels (see below). Dose addition is a single concept, but it has numerous mathematical representations. A general description of the history of dose addition is presented below, followed by highlights of recent advances to dose-addition modeling. For a more thorough review of work mentioned in the historical section and other methods developed prior to 1995, see the review by Greco et al. (1995).

9.3.1 Background

The first scientific publications on the effects of chemical combinations come from the field of pharmacology and its consideration of the joint action of drugs. Although often cited as providing the first description of the concept of dose additivity, Loewe and Muischnek (1926) refer to earlier work by Emil Bürgi and interpret the "Bürgi rule" as a reference to joint chemical effects described by an isobole (Bürgi 1912). Loewe and Muischnek also point out the inconsistent use of mixture-related terminology including synergism, antagonism, addition, and potentiation in the literature of the time; disagreement regarding these terms continues today (Kodell and Pounds 1991; Hertzberg and MacDonell 2002; Greco et al. 1992; Simmons 2013). In their seminal paper, Loewe and Muischnek (1926) present a spectrum of possible effects of a binary combination of chemicals through the use of isobolograms; in particular, they illustrate what we now call dose addition (then referred to as non-varying joint effects). This classic work presents a conceptual framework for addressing chemical combinations, but it does not describe applications of these concepts to experimental data.

The isobologram is a simple but effective graph for illustrating the concept of dose addition. Suppose that the horizontal and vertical axes of a graph represent the component doses of a binary mixture. Loewe and Muischnek proposed that the two chemicals are dose-additive if the isobole for a given response level is a diagonal line (with a negative slope) connecting the dose levels on the two axes that elicit that given response. For example, if chemical A elicits a 50% response at dose *a*, chemical B elicits a 50% response at dose *b*, and the straight line connecting points (*a*, 0) and (0, *b*) covers every dose combination of chemicals A and B that elicit a 50% response, then A and B are dose-additive at the 50% response level (Fig. 9.3). Loewe and Muischnek compared dose addition to other concepts: (1) deviation from dose additivity (e.g., greater than dose-additive, less than dose-additive); (2) independence of the mechanism that results in the same measured effect, i.e., exposure to each chemical results in the same apical outcome (e.g., death), but in each case, the outcome is achieved by a different underlying causative series of events (see Sect. 4.1); and (3) joint effects that are not elicited by either chemical alone but only by the combination (Fig. 9.3). Throughout their manuscript, Loewe and Muischnek described many key issues that continue to plague the field of mixture toxicology. They touched on the difficulty in distinguishing independent action of chemicals from "antagonism" (less than dose-additive interaction). They

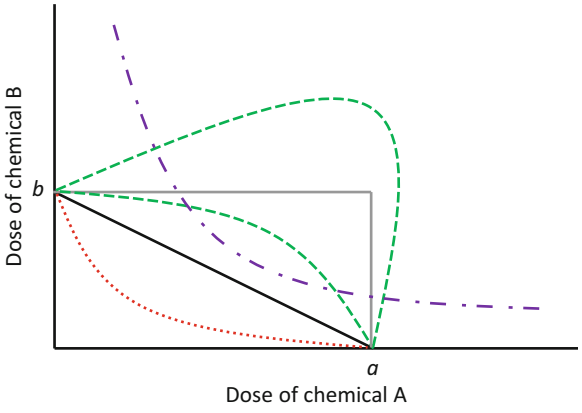

Fig. 9.3 Isobologram illustrating possible effects of a binary combination of chemicals (adapted from Loewe and Muischnek (1926)). Points *a* and *b* (with coordinates (*a*,0) and (0,*b*)) represent doses of chemicals A and B, respectively, that elicit equivalent effect levels (e.g., doses that elicit an effect that is 50% of the maximum response or the ED50). Each line/curve in the figure is an isobole, meaning that any combination of the concentrations of the two chemicals on that curve will result in the same effect level. The straight (black) diagonal line connecting points *a* and *b* is an isobole for two chemicals that are dose-additive. The dotted (red) curve represents a scenario where the combination of chemicals A and B results in effects that are greater-than dose-additive, and the two dashed (green) curves provide examples of less-than dose-additive scenarios. The dash-dot (purple) curve is an example of a combination of chemicals A and B eliciting effects not produced by any dose of either chemical alone. The angular isobole (perpendicular gray line segments) corresponds to a mixture where chemicals A and B act independently, and susceptibility to A is perfectly positively correlated with susceptibility to B (see Sect. 4.1)

also highlighted the difficulties in describing complex biological processes using simple mathematics, stating eloquently that "Biology does not know such simple and constant relationships." Furthermore, they acknowledged the need to incorporate experimental variability into evaluations of mixtures. Finally, Loewe and Muischnek suggested ways to quantify deviations from additivity. It is not surprising that this early, insightful work on mixtures has been so influential in shaping our current understanding and practice of mixture toxicology.

Bliss (1939), emerging from the fields of entomology and statistics and focusing on pesticide efficacy, provided another significant contribution to the field. Bliss described three possible joint action scenarios: "independent joint action" (a.k.a., independent action or response addition), "similar joint action" (dose addition), and "synergistic action" (encompassing greater-than- or less-than-additive scenarios). Although Bliss was primarily cited in reference to the origins of independent action (discussed in greater detail later in this chapter), his discussion of the joint action of chemicals was wide-ranging with many important observations. Regarding dose addition, he articulated more specific requirements for chemicals expected to exhibit dose-additive toxicity. In particular, Bliss argued that they should have the same mechanism of action ("act upon the same system of receptors" in producing the outcome of interest), identical susceptibilities (in a population context) to each chemical, and parallel dose-response curves, so that the only difference between chemicals would be in their potencies. Bliss noted that mixtures that are dose-additive "have a greater expected potency than those" that are response-additive. In other words, the dose-addition model predicts greater mixture toxicity than the independent-action model for the same component doses. In studies that compare results from dose-addition and independent-action models, there is a general trend of dose addition providing a higher predicted toxicity than independent action (Belden et al. 2007; Altenburger et al. 1996). However, there are exceptions to this observed trend (Cedergreen et al. 2008), and it has been demonstrated that the shapes of the individual chemical dose-response curves are critical in determining whether dose addition or independent action results in greater predicted toxicity (Christensen and Chen 1985; Drescher and Boedeker; 1995). To facilitate visualization of combination effects, Bliss proposed linearizing the dose-response data using a log-dose probit function and provided an example using data from experiments measuring mortality in house flies exposed to combinations of rotenone and pyrethrin (Bliss 1939). In that paper, Bliss focused on the "dosage-mortality curve" and thus defined toxic response only as the fraction of the exposed population dying. Other types of toxic response, such as continuous measurements of physiological function, are considered in later sections of this chapter.

Finney (1942) further developed many of the concepts proposed by Bliss (1939). In his work, Finney sought to more clearly define statistical methods for analyzing mixture data to obtain predictions based on an assumption of either independent action or dose addition. Finney demonstrated that the proposed methods can be applied to mixtures which contain more than two chemicals (Finney 1942). Drawing notable distinctions from Bliss (1939) and Finney (1942), Hewlett and Plackett published a body of work on models to describe joint action (Hewlett and Plackett

1957, 1959; Plackett and Hewlett 1952, 1963). In particular, they pointed out that individual chemicals need not have parallel dose-response relationships to be consistent with dose addition (Hewlett and Plackett 1959).

The next major contribution to the dose-addition literature was offered by Chou and Talalay in a series of highly cited papers (Chou and Talalay 1977, 1981, 1983, 1984). Their approach is based on the median effect principle, which was derived from the mass action law and has been applied mechanistically in biochemistry and pharmacology. For any given dose and corresponding fraction responding, the median effect principle focuses on two ratios: the ratio of the fraction responding to the fraction not responding and the ratio of the specified dose to the median effective dose (i.e., the ED50 or dose that elicits a 50% response). The first ratio is assumed to equal the second ratio raised to some power, where that power reflects the shape of the dose-response curve. The method relies on calculation of a combination index (CI), where CI $= 1$ indicates dose-additive effects, CI < 1 indicates greater-than-additive effects, and CI > 1 indicates less-than-additive effects. The CI is calculated by first fitting data from each chemical alone and a fixed ratio combination of the chemicals to the "median-effect equation" derived by Chou (1976), which is a rearrangement of the Hill equation, of which the Michaelis-Menten equation is a special case (Chou 2010). The CI value is then calculated from the resulting parameter estimates. Software was developed to facilitate the use of the approach, with the currently available CompuSyn (http://www.combosyn.com/index.html) representing the third generation of the software (Chou 2010). Greco et al. (1995) provide a thorough review and critique of the CI of Chou and Talalay, while Chou has more recently (2010) provided a review with recommendations for successful application of the method.

Contemporaneously with Chou and Talalay, Berenbaum presented another model for dose additivity (Berenbaum 1977, 1985, 1989). Berenbaum employed hypothetical "sham combinations" – combining a chemical with dilutions of itself – to articulate the dose-addition concept (Berenbaum 1989). In contrast to work by Loewe and Muischnek (1926) and Bliss (1939) described above, Berenbaum proposed that dose addition should be used as a general empirical method to describe the joint action of noninteracting chemicals, without requiring toxicological similarity among mixture constituents. Notably, Berenbaum stated that the only requirement is that the dose-response relationships for the individual chemicals are known over an "adequate range." The functions describing those relationships are unconstrained and are not required to be consistent across mixture constituents. On the topic of non-monotonic dose-response curves, Berenbaum posited that the proposed method can be used in cases of non-monotonicity observed in individual chemical dose-response curves while acknowledging that additional uncertainty may be associated with the analysis (Berenbaum 1985). He claimed that the proposed "general solution" put forth in his 1985 paper is a major departure from alternative models for describing combination effects, which require that "all the agents in the combination show similar dose-effect relations of the appropriate type" in the sense of similar functions and/or similarly shaped dose-response

curves. Many of those other approaches are based on or motivated by the interpretation of toxicological similarity as chemicals that are dilutions (concentrations) of each other and hence have the characteristics Berenbaum describes in his sham mixture example. He also contrasted his general solution with postulated requirements of sigmoidal dose-response curves for the median-effect equation, linear dose-response curves for effect summation, and simple exponential dose-response curves for independent action. While appropriate for the literature of 1985, those latter "requirements" are not considered necessary in current formulas based on independence (see Chap. 14).

The dose-addition model proposed by Berenbaum (1985) is based on the concept of linear isoboles and can be described mathematically by Eq. 9.1 for a given response level y:

$$\sum_{j=1}^{J} \frac{d_{j,y}}{\mathrm{ED}_{j,y}} = 1 \tag{9.1}$$

where J is the number of chemicals in the mixture, $d_{j,y}$ is the dose of chemical j in the mixture that produces response y, and $\mathrm{ED}_{j,y}$ is the dose of j alone that will produce response y (as estimated, e.g., from dose-response data for each individual chemical). For a binary combination, the equation simplifies to:

$$\frac{d_{\mathrm{A},y}}{\mathrm{ED}_{\mathrm{A},y}} + \frac{d_{\mathrm{B},y}}{\mathrm{ED}_{\mathrm{B},y}} = 1 \tag{9.2}$$

where $d_{\mathrm{A},y}$ and $d_{\mathrm{B},y}$ are the doses of chemicals A and B, respectively, in the mixture that elicit response y and $\mathrm{ED}_{\mathrm{A},y}$ and $\mathrm{ED}_{\mathrm{B},y}$ are the respective doses of chemicals A and B alone that elicit response y. Berenbaum referred to this equation as the "hyperplane theorem." Chemicals that individually do not produce an effect but that could increase or decrease the effect of other chemicals in a mixture can be incorporated into Eq. 9.1, as Berenbaum pointed out, by assuming that ED_j is infinite so that $\frac{d_j}{\mathrm{ED}_j}$ for that chemical is 0. At least one chemical must produce a nonzero response for Eq. 9.1 to hold. While chemicals that are dose-additive obey Eq. 9.1, a sum less than 1 indicates greater than dose-additive effects of the mixture, and a sum greater than 1 indicates less than dose-additive effects of the mixture. Berenbaum went on to demonstrate that the equation for dose addition (Eq. 9.1) can be rearranged to solve for the predicted mixture response *provided that all constituent chemicals display similar dose-response relationships* (Berenbaum 1985) – a restriction that is not needed for application of Eq. 9.1. Although Berenbaum's framework is relatively flexible compared to other approaches, limitations still exist. For example, mixture effects can only be calculated up to the maximum effect of the least effective constituent, thereby limiting the utility of this approach for mixtures containing partial agonists. Furthermore, Bosgra et al. have criticized the isobole approach advocated by Berenbaum and others based on the possibility of noninteracting chemicals that display nonlinear isoboles (Bosgra et al. 2009).

Nevertheless, the work of Berenbaum has provided the foundation for many subsequent efforts to refine predictive models for mixture toxicity based on an assumption of dose additivity.

9.3.2 Summary of Select Dose-Addition Models

This section discusses approaches that have been developed more recently. Altenburger and colleagues have made significant contributions to the dose-addition literature from the field of ecotoxicology (Altenburger et al. 1990, 2000, 2004; Faust et al. 2001). Their work systematically evaluated mixtures of chemicals with similar and dissimilar mechanisms of action in order to better understand the joint action of combinations of environmental chemicals. Their overarching goal appears to be the development of general principles that can improve our ability to predict mixture effects based on dose-response relationships for component chemicals. To this end, they have adapted established dose-addition (and independent-action) concepts and applied them to a number of different mixtures. Their work evaluates which additivity models are most appropriate for estimating the toxicity of different mixtures with known mechanisms of action. For example, they test the hypothesis that chemicals with the same mechanism of action will better conform to a model of dose addition than to a model of independent action. Their approach represents a practical application of the dose-addition principles described by Berenbaum. First, various dose-response functions (e.g., probit, logit, Weibull) are fit to data from each individual chemical to identify a separate "best fit" function for each chemical (i.e., there is no requirement for a shared function among constituents). This feature is relatively uncommon among dose-addition models, which typically use a common function to describe all individual chemical dose-response curves. Next, the proportion of each individual chemical in the mixture and the dose corresponding to a designated response level derived from the "best fit" function serve as input for calculating the predicted mixture dose corresponding to that effect level. For mixtures of known and fixed composition, Altenburger et al. (2000) rearrange the variables in the equation proposed by Berenbaum (Eq. 9.1) to solve for the dose of the mixture that would elicit a given effect level y:

$$\mathrm{ED}_{\mathrm{mix},y} = \left(\sum_{j=1}^{J} \frac{q_j}{\mathrm{ED}_{j,y}} \right)^{-1} \tag{9.3}$$

where $ED_{\mathrm{mix},y}$ and $ED_{j,y}$ are the doses of the mixture and individual chemical j, respectively, expected to elicit a designated effect level y, and q_j is the fraction of chemical j in the mixture. The mixture dose $ED_{\mathrm{mix},y}$ is calculated for a range of y values spanning approximately 1–99% of the maximal response. This results in articulation of the predicted dose-response relationship for the mixture. As with the

original Berenbaum approach, predictions can be made only up to the lowest maximum effect level among the individual constituents and down to the highest minimum effect level. This limitation is not ideal for mixtures containing constituents that display incomplete efficacy (i.e., partial agonists). Generally, Altenburger et al. compare predictions of mixture toxicity based on dose addition, along with predictions based on independent action as a second reference curve, to observed mixture toxicity over a range of doses.

The method first described by Altenburger et al. (2000) has been applied by many other groups to evaluate diverse environmental mixtures in a wide array of test systems from in vitro estrogenicity assays (Silva et al. 2002; Rajapakse et al. 2004; Payne et al. 2000) to in vivo rodent toxicity studies (Metzdorff et al. 2007). Although the essentials remain consistent, the approach has been applied with various modifications and increasing sophistication. For example, the collection of functions from which the "best fit" to individual chemicals is selected may differ among research groups. Additionally, bootstrap methods have been used to incorporate statistical uncertainty into model predictions to more rigorously evaluate the differences between predicted and observed dose-response relationships (Metzdorff et al. 2007). Finally, Scholze et al. have adapted the method to assess mixtures containing constituents that display partial efficacy (Scholze et al. 2014).

The work of Gennings and colleagues offers another significant body of literature providing methods for detecting departure from dose additivity of chemical mixtures (Casey et al. 2004; Gennings et al. 1997, 2004a, b). A defining hallmark of this work is in the statistical comparison of predicted to observed mixture effects using the whole dose-response curve instead of, or in addition to, a point-by-point comparison. Models developed by Gennings et al. have been applied to a diverse array of mixtures and testing paradigms. A detailed description of this and related statistical approaches for dose additivity can be found in Chap. 11.

Another example of adapting Berenbaum's dose-addition principles comes from Howard and Webster, who offer a novel approach to assessing mixtures containing constituents with partial efficacy (e.g., partial agonists) (Howard and Webster 2009). In contrast to the methods developed by Altenburger et al., this approach incorporates a single model (3-parameter Hill model) for all individual chemicals. The slope (i.e., Hill coefficient) for each chemical is assumed to be 1. Howard and Webster's equation for the "generalized concentration addition" model for a given response y is:

$$\sum_{j=1}^{J} \frac{d_{j,y}}{f_j^{-1}(y)} = 1 \tag{9.4}$$

where $d_{j,y}$ is the dose of chemical j in the mixture that produces response y and f_j^{-1} is the inverse of the dose-response function f_j for chemical j. Compared to the Berenbaum formula of Eq. 9.1, Eq. 9.4 is simply a change in notation: replacing $\mathrm{ED}_{j,y}$ by $f_j^{-1}(y)$. The novelty arises because Howard and Webster formally define the inverse function for partial agonists on the full range of responses possible for

the full agonist, revising the usual definition where each inverse function is defined only on the range of responses for that individual chemical. This revised definition eliminates some dose-response models from consideration. When the dose-response model is a Hill model with a slope equal to 1, this revised definition assigns "negative" doses of a partial agonist to response levels beyond that partial agonist's maximal response and, for binary mixtures of a partial and a full agonist, leads to linear isoboles with positive slope at those response levels. If the assumption about the Hill slope is tenable, this approach is particularly well-suited for assessing dose additivity for mixture data from receptor-based in vitro assays that frequently include partial agonists (Howard et al. 2010; Hadrup et al. 2012, 2013).

9.3.3 Application of Dose-Addition Modeling in Toxicology: Male Reproductive Tract Development

Endocrine-disrupting chemicals have been the focus of a large body of mixture research that uses dose-addition modeling as a tool to explore the joint action of potentially co-occurring chemicals. This line of research is motivated by multiple factors. First, there is concern that a number of reproductive tract malformations and pathologies (e.g., testicular dysgenesis syndrome) are increasing in certain populations; exposure to environmental contaminants has been implicated in this trend (Main et al. 2010). Second, the U.S. National Health and Nutrition Examination Survey (NHANES) (Buttke et al. 2012) and some international biomonitoring efforts (Frederiksen et al. 2014) have demonstrated that people are routinely exposed to numerous chemicals that have endocrine-disrupting potential. Third, the period of reproductive tract development represents a particularly sensitive window. Fourth, the joint effects of endocrine-disrupting chemicals that act at different points in complex signaling pathways are not fully understood. Fifth, knowledge of whether chemicals display dose-additive or greater than dose-additive toxicity provides information useful for the risk assessment of chemical mixtures.

Research groups at the U.S. EPA led by Gray (Rider et al. 2010, 2008) and in Europe led by Hass and Kortenkamp (Christiansen et al. 2009; Metzdorff et al. 2007; Ermler et al. 2011) have used dose-addition modeling to understand how individual endocrine-disrupting chemicals act jointly to disrupt male reproductive tract development. Both groups have identified individual environmental chemicals (e.g., pesticides, herbicides, plasticizers, personal care product ingredients) that disrupt male reproductive tract development through different mechanisms (e.g., androgen receptor antagonism, disruption of steroidogenesis). The general hypothesis developed by these researchers is that chemicals that share common key events in their respective adverse outcome pathways (see Chap. 7 for detailed information on using adverse outcome pathways to prioritize mixtures for study), or that simply share a common adverse outcome, will adhere to predictions based on dose

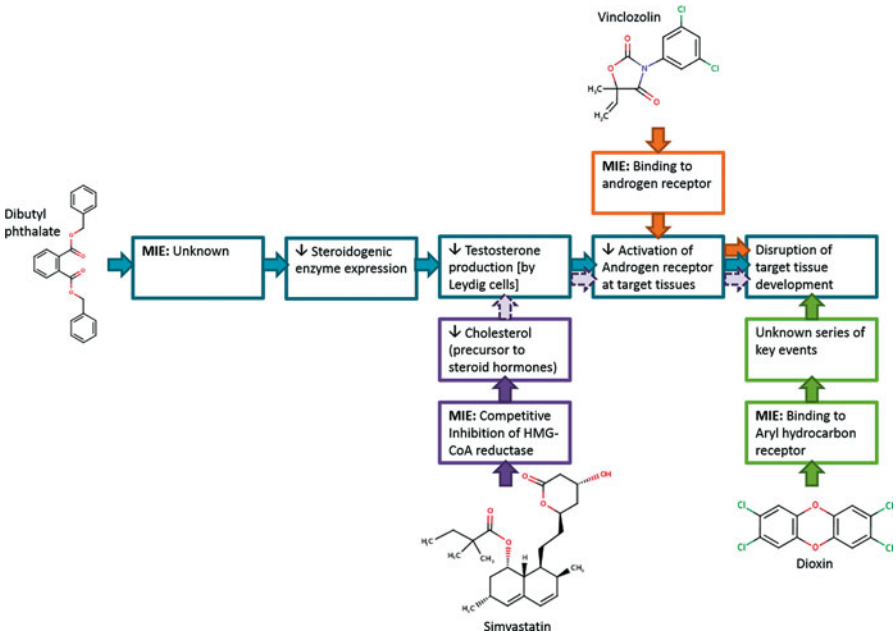

Fig. 9.4 Hypothetical adverse outcome pathway network for four chemicals that disrupt male reproductive tract development. Each chemical displays a unique molecular initiating event (MIE). Solid arrows represent pathways with substantial scientific support, while lighter arrows with dashed lines represent hypothesized pathway interactions. Some of the key events are unknown, including the molecular initiating event for phthalates and the intermediate steps for dioxin

addition. In other words, a convergence of pathways at or near the manifestation of the adverse outcome is hypothesized to result in combined effects that are dose-additive.

Figure 9.4 provides a visual representation of the general hypothesis. In this example, three chemicals (vinclozolin, dibutyl phthalate, and dioxin) elicit reproductive toxicity via different mechanisms of action (i.e., they activate different molecular initiating events). The fungicide vinclozolin binds to the androgen receptor thereby blocking the action of androgens (e.g., testosterone, dihydrotestosterone) responsible for normal development of male reproductive tissue. Dibutyl phthalate, a plasticizer, decreases the availability of testosterone. Vinclozolin and dibutyl phthalate share a common adverse outcome of disrupting the development of male reproductive organs in rats exposed in utero. Though not all of these chemicals elicit the same suite of effects, some effects are overlapping (e.g., disruption of epididymal development). The biological underpinning of applying the dose-addition hypothesis to binary combinations of vinclozolin and dibutyl phthalate is that the target tissue (i.e., epididymal tissue) does not recognize whether the decrease in activated androgen receptor is due to receptor antagonism

or decreased androgen; the tissue only recognizes the total decrease in activated receptors. Even though the specific mechanisms are different, their convergence supports the use of dose addition. Multiple studies with combinations of chemicals with the mechanisms described above have shown that predicted mixture responses based on dose addition are similar to observed mixture responses, supporting the hypothesis (Rider et al. 2008, 2010).

The hypothesis of dose addition is less clearly applied to the addition of dioxin to the binary mixture. Dioxin's mechanism of action is not known, and its effect on epididymal development is less well-defined. To expand the dose-addition hypothesis to the ternary mixture, dose-response data from the three chemicals that exhibit reproductive toxicity could be used to calculate expected effects of a three-chemical mixture based on an assumption of dose additivity. These predictions could then be compared to empirical data generated from testing the defined mixture.

A fourth chemical, simvastatin, is known to disrupt cholesterol synthesis, but it does not exhibit similar effects on reproductive endpoints. The hypothesis of dose addition could be used to investigate the joint action of simvastatin with the other three reproductive toxicants. Here, the assumption would be that simvastatin would not contribute to the toxicity of the three-chemical mixture (i.e., its contribution to dose-additive toxicity would be 0). A greater than dose-additive or less than dose-additive result would indicate that, although simvastatin does not directly induce the adverse outcome, it does alter the toxicity of one or more of the three chemicals that act through the androgen receptor-mediated adverse outcome pathway (Fig. 9.4). Mechanistic studies would then be required to investigate the hypothesis that decreased cholesterol could lead to a decrease in testosterone sufficient to contribute to downstream toxicity.

In addition to work aimed at exploring the dose additivity of antiandrogenic mixtures, similar work looked at estrogenic mixtures (Silva et al. 2002; Rajapakse et al. 2004) and at thyroid-disrupting mixtures (Crofton et al. 2005). Data from experiments like those described above help to inform cumulative risk assessment. For example, such studies contributed to the decision by a National Academy of Sciences panel to recommend to the U.S. EPA that reproductive toxicant phthalates, as well as other antiandrogenic chemicals, should be included in cumulative risk assessments (National Research Council 2008).

9.4 Independent Action

As with many terms in mixture toxicology, "independent action" is not used consistently throughout the literature. In particular, "independent action" has been used interchangeably with "independent joint action," "response addition," and "Bliss independence." Here, the term "independent action" was deliberately selected. Although "response addition" provides a convenient counterpart to the term "dose addition," it does not adequately convey the underlying concept. First,

the terms "response" and "addition" can be synonyms of "effect" and "summation," respectively. In this chapter, the concepts of "response addition" and "effect summation" are carefully defined so they are not equivalent and lead to different prediction formulas for the combination exposure (see detailed discussion later in chapter). Second, independent action does not necessarily lead to a simple addition of responses. Although independent action and independent joint action have both been used widely, a preference for simplicity favored the use of independent action herein.

9.4.1 Background

The first description of independent action is generally attributed to Bliss (1939). The foundational biological premise of independent action is that individual chemicals act through distinct, non-interfering pathways to arrive at an apical response (see Sect. 2 for further discussion). In a binary mixture with independent action, the presence of chemical B does not influence the dose of or the response to chemical A and vice versa. The work of Bliss is based on pesticides and refers specifically to quantal responses, those involving some measurable two-category event (generically, occurrence/nonoccurrence, presence/absence, or yes/no, e.g., death, presence of a particular tumor at sacrifice, or body weight less than 18 g). Quantal responses are characterized by the probability that the event occurs as estimated by the fraction of the population (or sample) that experiences the event. The equation for calculating mixture response under independent action most often cited in the toxicology literature is:

$$p_{\text{mix}} = 1 - \prod_{j=1}^{J} \left(1 - p_j\right) \tag{9.5}$$

where p_{mix} is the probability of the event occurring in response to a given dose of the mixture and p_j is the corresponding probability for chemical j alone at the same dose as it is present in the mixture. Equation 9.5 is a consequence of an elementary probability calculation that assumes that the occurrence of the event in response to an individual chemical is independent across the chemicals. The probability that the event does not occur in response to the mixture is $1–p_{\text{mix}}$. For the event not to occur in response to the mixture means that the event cannot occur in response to any of the individual chemicals (if one or more of the individual chemicals at their dose in the mixture had elicited the event, then the mixture dose would have elicited the event). The non-occurrence probability for chemical j is $1–p_j$. Under the independence assumption, the probability that no single chemical elicited the event is the product of their individual non-occurrence probabilities, namely, $\prod_{j=1}^{J} \left(1 - p_j\right)$. Equating $1–p_{\text{mix}}$ to this product and solving for p_{mix} yields Eq. 9.5.

For a binary combination of chemicals, the formula simplifies to:

$$p_{\text{mix}} = p_{\text{A}} + p_{\text{B}} - (p_{\text{A}} p_{\text{B}}) \tag{9.6}$$

where p_{A} and p_{B} are the event probabilities for individual chemicals A and B, respectively. Because the fraction of individuals who respond both to chemical A and to chemical B is included twice in the sum $p_{\text{A}} + p_{\text{B}}$, the term ($p_{\text{A}}$ p_{B}) must be subtracted to compensate for the double counting. Bliss noted that this formula corresponds to Abbott's formula, which is used in entomology to distinguish between mortality attributable to pesticide application or to natural causes (Abbott 1925).

Bliss pointed out that the underlying assumption about independent action leads to different predictions of the mixture response depending on the "correlation in susceptibility" to the distinct mixture constituents. Imagine a population in which individuals exhibit a range of susceptibilities or tolerances (i.e., the exposure level of a given chemical required to elicit the event differs among individuals). In addition, suppose that each individual has a separate susceptibility to each distinct chemical in a mixture. In a population of individuals, the susceptibilities to distinct chemicals may be correlated or not. Limiting himself to two chemicals, Bliss considered three possibilities: no correlation (no relationship between susceptibilities), perfect positive correlation (ordering of individuals' susceptibilities is the same for both chemicals), and some intermediate degree of positive correlation. Equations 9.5 and 9.6 correspond to independent action with uncorrelated susceptibilities.

In Fig. 9.3, the angular isobole (perpendicular gray line segments) corresponds to the case of independent action with perfect positive correlation among susceptibilities. That special case is likely to be extremely rare for real mixture exposures but merits inclusion here because it shows up in mixture literature, usually without explanation. When the susceptibility correlation is positive but less than perfect, the isobole would have a different shape that depended on the shapes of the dose-response functions for the two chemicals. The concept of independent action, regardless of the correlation among susceptibilities, properly applies only to quantal responses.

Plackett and Hewlett (1948) also contributed to describing the concept of independent action put forth by Bliss and went into further detail on potential correlation in susceptibilities. In addition to the options described by Bliss, Plackett and Hewlett considered perfect negative correlation (the ordering of individuals' susceptibilities to one chemical is exactly the reverse of their susceptibilities to the other), as well as intermediate degrees of negative correlation. For perfect negative correlation of susceptibilities, the probability of response to the mixture is given by:

$$p_{\text{mix}} = \min\,(p_A + p_B,\ \ 1), \tag{9.7}$$

$$p_{mix} = p_A + p_B - p_A p_B$$

.0199 = .01 + .01 = .02 .01 x .01 = .0001
.0494 = .02 + .03 = .05 .02 x .03 = .0006
.0976 = .06 + .04 = .10 .06 x .04 = .0024
.1904 = .08 + .12 = .20 .08 x .12 = .0096

Fig. 9.5 Example of calculating mixture effects at low individual effect levels (<15%) when assuming independent action. The product $p_A p_B$ is small and does not appreciably change the sum $p_A + p_B$

which reflects the constraint that a probability cannot exceed 1. In particular, the proportion responding to the mixture cannot exceed 1, regardless of the proportions responding to the individual constituents.

Effect summation is considered in the next section as a separate approach from independent action, though there is an overlap between the two concepts. The usual effect-summation formula for a binary mixture is:

$$p_{\text{mix}} = p_{\text{A}} + p_{\text{B}} \tag{9.8}$$

and corresponds to Eq. 9.7 (for independent action with perfect negative correlation of susceptibilities) if the sum of p_A and p_B does not exceed 1, and it approximates Eq. 9.6 (for independent action with no correlation of susceptibilities) if the response probabilities are small enough that the product of p_A and p_B is negligible (see, e.g., Fig. 9.5).

Assessing correlations among pairs of susceptibilities is not trivial with two chemicals and becomes unmanageable with more than two chemicals. Furthermore, if chemicals are indeed working through different (i.e., independent) mechanisms, perfect correlation (either positive or negative), though instructive for illustrating the limiting cases, seems unrealistic. The difficulty in assessing these correlations coupled with the implausibility of perfectly correlated susceptibilities could explain why correlation of susceptibilities is not discussed widely in current mixture literature and why most researchers default to Eq. 9.5, which corresponds to the assumption of uncorrelated susceptibilities. Unless specifically stated, this chapter will take "independent action" to mean "independent action with uncorrelated susceptibilities" as in Eqs. 9.5 and 9.6.

9.4.2 Application of Independent Action

As with many concepts in mixture toxicology, the application of independent action has evolved over time to scenarios beyond the original scope. Most importantly, independent action is currently used to predict a variety of biological responses that

do not reflect the initial definition, derived from probabilities of events. Equations 9.5 and 9.6 are specific calculations for the probability of co-occurrence of statistically independent events; consequently, they properly apply to the probability that the event occurs or the proportion of the population that experiences the event, quantities that must fall between zero and one. Quantal response data that meet the original definition of independent action include mortality and the proportion of animals within a dose group that displays a certain characteristic. Continuous endpoints (e.g., growth, organ weight, hormone levels, magnitude of gene expression changes) generally do not lie between zero and one and are not interpretable as probabilities; consequently, they fall outside of the scope of that definition. If the continuous response (y) has predefined minimal and maximal possible values (y_{min}, y_{max}), then it can be rescaled to lie between zero and one (equivalently, 0% and 100%) by transforming to $(y-y_{min})/(y_{max}-y_{min})$. Even such rescaled responses, however, are not typically interpretable as "probabilities." For example, knowing that an exposed rat lost 40% of its body weight says little about the probability of that response. Thus, the use of Eq. 9.5 with continuous responses, even after transformation to a scale that looks like a probability, is not supported by the underlying probability argument that justifies Eq. 9.5. Furthermore, while the $1–p$ term in Eq. 9.5 is clearly interpreted as the probability of non-occurrence (e.g., if the event is death, then non-occurrence is survival), we do not know of any analogous interpretation of the corresponding $1–y$ term. Consequently, application of Eqs. 9.5 or 9.6 with quantal responses has no similar theoretical basis as a benchmark for independent action when applied to continuous responses. In other words, there is no reason to believe that chemicals that act independently will obey Eqs. 9.5 or 9.6 when the ps are replaced by ys, even rescaled ys. Nevertheless, despite this serious theoretical shortcoming, using the independent-action formula as an empirical model with continuous responses is widely practiced and may have utility.

Early discussions of applying independent action for binary mixtures to continuous response data emphasized differences in response measures (Muska and Weber 1977). While quantal data show fractions of the population exceeding toxicity thresholds, continuous data show average measured response intensities. Thus, the concept of susceptibility correlation is not appropriate for continuous data and formulas like Eq. 9.7 cannot be used. Instead, estimates of combined responses are simple sums of component response intensities (see section 9.5 for more about effect summation). More recent instances of applying independent action to continuous endpoints employ Eq. 9.5. Examples in the literature of the application of independent action to continuous data include in vitro enzyme activity (Froment et al. 2016), degree of hypopigmentation in zebra fish (Schmidt et al. 2016), and organ weights in rat pups following in utero exposure (Rider et al. 2008), among others.

An example can be used to illustrate the different applications discussed above. Imagine a study where pregnant females are exposed to different doses of chemical A or chemical B alone and their pups are evaluated for responses. The two endpoints of interest are pup mortality (Table 9.1) and pup weight at birth (Table 9.2). For the mortality endpoint, the percent of pups who died is the response

Table 9.1 Hypothetical data to illustrate application of independent-action model

Chemical A		Chemical B		
Dose (mg/kg)	Mortality (%)	Dose (mg/kg)	Mortality (%)	Predicted mixture response under independent action (%)
0	0	0	0	0
2	3	50	5	8
4	10	100	20	28
8	50	200	45	73
16	95	400	75	99

used in the model. Therefore, Eq. 9.5 could be used to calculate the expected response of a binary combination of the two chemicals (at each of the dose pairings in a given row of the table) under an assumption of independent action as in the following example:

$$4\ \text{mg/kg A} + 100\ \text{mg/kg B} : 0.10 + 0.20 - (0.10^{*}0.20) = 0.30 - 0.02 = 0.28 = 28\%\text{mortality}$$

In contrast, pup weight does not easily fit into the probability-based equation. As opposed to mortality, mean pup weight represents an average value of a continuous variable. Before discussing the data transformation that is required to apply the model, it is important to address the rationale for applying the independent-action model to these data. There are two possible arguments. First, the model can be viewed as a strictly empirical tool, offering a point of comparison for dose-addition predictions and observed mixture data. Second, and more problematically, one could argue that individual chemicals are acting through different biological mechanisms to disrupt a common system (e.g., development) that has real biological limits, justifying a meaningful rescaling to 0–100%. In the current example, pup weight data could be converted to % decrease from control (but even this more meaningful rescaling is not interpretable as providing probabilities, so it should just be treated as an empirical model). The mean weight in the control group could serve as a maximum response level (y_{max}) – though using observed means can lead to anomalies (e.g., negative percentages which are not between 0 and 100%) (Table 9.2). As a minimum response level (y_{min}), 0 g could serve as a default minimum (pups cannot weigh less than 0 g), or, assuming pups are not viable below some nonzero weight, the average of "lowest pup weights" achieved in previous work could serve as a more biologically-based minimum. If all pup weights are rescaled using the control value (e.g., the average weight of the combined control animals from chemicals A and B, which is 6.3 g in this example) as y_{max} and the minimum viable weight (3.0 g in this example) as y_{min}, then the data can, in principle, be converted to a 0–100% response scale (Table 9.2). These converted data can then be used to make predictions based on the independent-action equation, as described above for mortality.

Table 9.2 Conversion of continuous data to a 0–100% scale for use in Eq. 9.5 to predict mixture responses assuming independent action

Chemical A			Chemical B			Predicted mixture response under independent action
Dose (mg/kg)	Mean pup weight at birth (g)	% Decrease from control	Dose (mg/kg)	Mean pup weight at birth (g)	% Decrease from control	% Decrease from control
0	6.2	3.0	0	6.4	−3.0	0.1
2	5.9	12.1	50	6.5	−6.1	6.7
4	5.7	18.2	100	5.7	18.2	33.1
8	4.5	54.5	200	5.1	36.4	71.1
16	3.9	72.7	400	4.2	63.6	90.1

[a]A minimum response of $y_{min} = 3.0$ g was assumed when calculating the % decrease from control, which is defined as $100 \times (y_{max} - y)/(y_{max} - y_{min})$, where y_{max} is the average control response of 6.3 g

In the past, debate has centered around which definition of additivity – dose addition or independent action – should serve as the default approach for describing the baseline prediction from which interactions should be measured (Greco et al. 1992). This argument has largely faded, with many researchers instead opting to include both dose-addition and independent-action predictions as referents for assessing tested mixtures (Rider et al. 2008; Olmstead and LeBlanc 2005; Christiansen et al. 2009; Gregorio et al. 2013; Altenburger et al. 2000; Qin et al. 2011). Including both formulas is usually done when there is inadequate biological information about the potential joint toxicity. It is typically held that independent action is appropriate for chemicals that act through different adverse outcome pathways, while dose addition is favored for chemicals that exhibit similar mechanisms of action. However, individual chemical adverse outcome pathways are not always known. Furthermore, instead of a priori classification of individual chemicals and selection of an appropriate model, the fit of observed mixture data to either dose-addition or independent-action models has been used to support arguments regarding similarity/dissimilarity of mechanism(s) among mixture constituents (Rider and LeBlanc 2006; Froment et al. 2016; Faria et al. 2016). In many cases, however, dose addition and independent action yield similar or even indistinguishable predicted mixture effects, particularly when the response (as a probability) is very small or when the dose-response curve is close to linear (Thienpont et al. 2013; Kortenkamp and Altenburger 1998; Cedergreen et al. 2008).

Several studies have addressed the hypothesis that chemicals with distinctly different mechanisms of action are better fit by a model of independent action than by one of dose addition (Backhaus et al. 2000, 2011; Ermler et al. 2014; Villa et al. 2012; Faust et al. 2003; Hermens and Leeuwangh 1982; Martin et al. 2009; Baylay et al. 2012; Cedergreen et al. 2008); however, few of these studies found that

independent action consistently provided a significantly better fit than dose addition (Backhaus et al. 2000; Faust et al. 2003).

Some of the most highly cited work on independent action comes from Backhaus and colleagues (Faust et al. 2003; Backhaus et al. 2000). These foundational studies set out to carefully investigate the joint action of chemicals with strictly dissimilar mechanisms of action. They tested mixtures of 14 and 16 constituents with the microtox assay (measuring disruption of the respiratory process of the marine bioluminescent bacteria *Vibrio fischeri*) and algal toxicity, respectively. In both studies, they found that independent action accurately predicted mixture toxicity, while dose addition overestimated the responses of the mixture, across the range of mixture dilutions along fixed-ratio rays with constituents present at equipotent concentrations (e.g., constituents present at the ratio of their concentrations eliciting a 50% effect when tested individually).

In the majority of papers evaluating chemicals with dissimilar mechanisms of action for adherence to independent-action predictions, the results are much less clear-cut. In a series of experiments assessing the effects of binary mixtures of dissimilar chemicals on *C. elegans* egg production, Martin et al. (2009) found some cases where independent action accurately predicted observed mixture effects and other cases where it over- or underestimated observed mixture effects. In another example, Ermler et al. (2014) found that a mixture of four chemicals with dissimilar mechanisms of action resulted in genotoxic effects that fell between independent-action and dose-addition predictions. Similarly, Petersen et al. (2014) observed a dose-dependent switch from independent action at low concentrations to dose addition at higher concentrations for a mixture of eight chemicals with diverse mechanisms of action on the growth rate of marine algae. Baylay et al. (2012) pursued a mechanistic understanding of the combination of two dissimilar chemicals using metabolomic profiling of earthworm tissue following treatment with nickel, chlorpyrifos, and a combination of the two. They concluded that while the measured effect of the mixture on reproduction was greater than that predicted by independent action, the hypothesis of dissimilar mechanisms of action was supported by the finding that the metabolomic profile for the mixture was intermediate between the unique profiles for nickel and chlorpyrifos alone, confirming their dissimilar activity and indicating that they both contributed to the mixture effect.

9.4.3 Challenges with Independent Action

As indicated by the name, independent action is predicated on the assumption that the individual chemicals within the mixture operate independently; however, the exact nature of the biological independence required to make this assumption plausible is usually not clearly articulated. Throughout the independent-action literature, there are references to independence at the level of mechanism of action (adverse outcome pathway), target tissue, or target system. There are two important points to keep in mind. First, considering the complexity of biological systems, it is

unlikely that the mechanisms of action will be strictly dissimilar. Second, many environmental chemicals can have more than one mechanism of action.

As alluded to above, independence of action is likely influenced by the chemical constituents, endpoint, and model system of interest. Therefore, one can hypothesize that experiments that include chemicals with specific and distinct mechanisms of action and simple model systems are more likely to produce observed results consistent with independent-action predictions. Conversely, chemicals with nonspecific mechanisms of action, common among environmental contaminants, and complex systems (e.g., carcinogenesis in a rodent) might be more likely to involve interactions among biological signaling pathways and less likely to conform to predictions based on an assumption of independent action. Hermens and Leeuwangh (1982) discuss some of these considerations in their studies with chemicals that display both specific and nonspecific mechanisms of toxicity. For example, both Hermens and Leeuwangh (1982) and Könemann (1981) discuss the concept that the primary mechanisms of constituent chemicals could act independently, while a lesser (secondary) narcotic mechanism present in all organic chemicals could contribute in a dose-additive manner to toxicity, resulting in observed mixture toxicity that exceeds independent-action predictions.

9.5 Effect Summation

As mentioned earlier (Sect. 4.1), under certain conditions (e.g., low effect levels), effect summation can be applied directly with quantal response data to approximate predictions under independent action. Nevertheless, some investigators have applied effect summation beyond quantal responses to continuous responses, even without prior conversion to a 0–100% scale. Avoiding such conversion is particularly useful when the responses being measured are not readily amenable to it. Of course, the sum of effects, whether rescaled or not, can exceed the 100% limit for probabilities or the experimentally determined maximum effect level for continuous responses – a characteristic that is often cited as a fatal flaw of the approach (see below for a discussion of the limited application of effect summation). The general equation for effect summation is:

$$Y_{\text{mix}} = \sum_{j=1}^{J} Y_j \tag{9.9}$$

where Y_j is the response elicited by chemical j alone. For a binary mixture, the equation simplifies to:

$$Y_{\text{mix}} = Y_{\text{A}} + Y_{\text{B}} \tag{9.10}$$

where Y_{A} and Y_{B} represent the responses elicited by chemicals A and B, respectively, when given alone.

9.5.1 Application and Challenges of Effect Summation

In toxicological studies, effect summation is often cited as an inappropriate approach for assessing mixtures because of a lack of biological plausibility at high effect levels (Boedeker and Backhaus 2010). A primary example of this implausibility is that an effect-summation model can produce response predictions beyond the natural response boundaries. In contrast, for independent action, the laws of probability ensure that the predicted probabilities satisfy the natural boundary constraints. Thus, effect summation is frequently discounted as an appropriate tool in toxicology because a biological plausibility threshold can be exceeded. Nevertheless, effect summation does have one important advantage over independent action: there is no requirement to convert the individual data, so they can be combined across chemicals in raw form. Therefore, effect summation could be used as a benchmark, if researchers acknowledge its limitations.

The example described in Sect. 4.2 can be used to illustrate the advantages and disadvantages of effect summation compared to independent action. In Table 9.3, it is apparent that the responses can be added together without converting the data. However, at the highest dose of the mixture (16 mg/kg chemical A + 400 mg/kg chemical B), the predicted effect is a 4.5 g decrease in pup weight. This decrease would result in pup weights around 2.0 g, well below the 3.0 g that was thought to be the minimum that is biologically plausible (see independent-action example above). This again emphasizes the need for a biologically-based limit on the predicted effect. Because most effect measures are surrogates for a complex physiological process, many effect limits are empirically derived, including limits on what values are considered "normal" or "healthy." At those highest doses in Table 9.3, the pups are of such low weight as to be biologically compromised. For example, at the highest dose of chemical A (16 mg/kg), the pup weight loss is 37%, well in excess of the common limit of 10% (Chapman et al. 2013). Furthermore,

Table 9.3 Predictions of mixture responses based on effect summation

Chemical A			Chemical B			Effect-summation prediction
Dose (mg/kg)	Mean pup weight (g)	Decrease in pup weight (g) from control[a]	Dose (mg/kg)	Mean pup weight (g)	Decrease in pup weight (g) from control[a]	Decrease in pup weight (g) from control[a]
0	6.2	0.1	0	6.4	−0.1	0
2	5.9	0.4	50	6.5	−0.2	0.2
4	5.7	0.6	100	5.7	0.6	1.2
8	4.5	1.8	200	5.1	1.2	3.0
16	3.9	2.4	400	4.2	2.1	4.5

[a]The control response for calculating decrease in weight was the average of the zero-dose responses for both chemicals, namely, 6.3 g

they likely have some physiological or biochemical processes that are no longer functioning normally, calling into question the purpose of the predictive model based on one measured effect.

9.6 Integrated Addition

Integrated addition is a more recent development in the mixture literature and represents a combining of concepts from dose addition and independent action to accommodate mixtures that contain constituents with similar and dissimilar adverse outcome pathways (Rider and LeBlanc 2005; Teuschler et al. 2004; Altenburger et al. 2005). First, chemicals are grouped into toxicologically similar groups (see discussion in Sect. 2). Next, the total response for each group is calculated using a dose-addition method. This response must be a measure of probability (see Sect. 4.2 for concerns). Finally, the responses from the different groups are combined using an independent-action approach (Figs. 9.6 and 9.7). The goal driving development of the integrated-addition approach was to refine predictions of mixture toxicity based on known mechanisms of action.

9.6.1 Application of Integrated Addition

In one of the first papers describing an integrated-addition approach, a ternary mixture of two organophosphate pesticides (malathion and parathion) and the

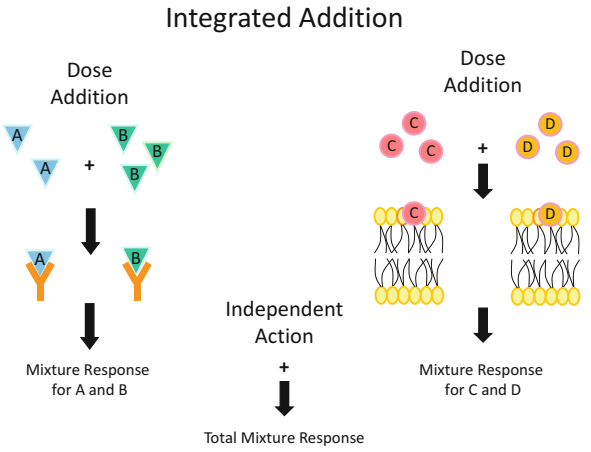

Fig. 9.6 Representation of integrated addition. In this example, chemicals A and B share a common mechanism of action (binding to a receptor to elicit a downstream effect), as do chemicals C and D (interfering with lipid membranes). A dose-addition model can be used to calculate expected mixture effects for each of the two mechanism-based groups. The responses of each of the mechanism-based groups can then be combined using an independent-action model

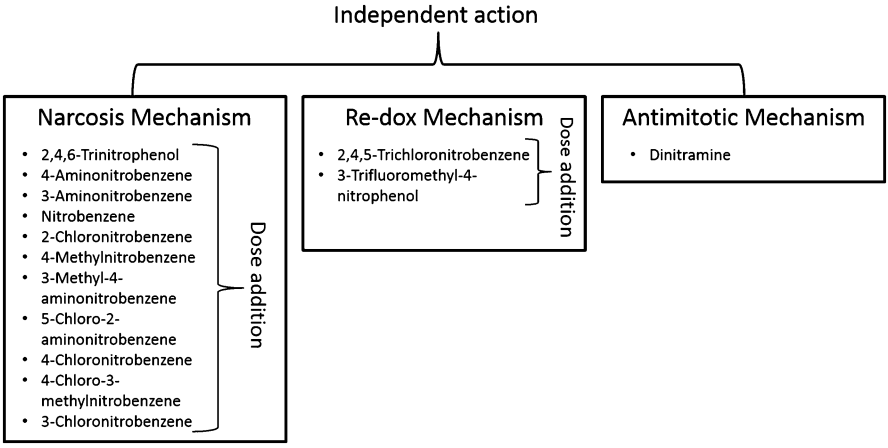

Fig. 9.7 Example of an application of the integrated-addition concept to a 14-chemical mixture of nitrobenzenes (Altenburger et al. 2005)

pesticide synergist piperonyl butoxide was assessed for its joint effects on immobilization of the crustacean *Daphnia magna* (Rider and LeBlanc 2005). Malathion and parathion inhibit acetylcholinesterase activity. Their joint action was estimated using dose addition. Next, piperonyl butoxide effects were combined with the dose-additive effects of malathion and parathion using independent action (Eq. 9.5). An interaction coefficient was also added to the equation to account for the interaction between organophosphates and piperonyl butoxide. (Quantitative interaction information is rarely available, and the modification of models to incorporate interactions is beyond the scope of this chapter.)

In another early effort to combine dose addition and independent action, Altenburger et al. (2005) used various mechanism-based grouping strategies to compare modeled predictions to observed algal toxicity of a 14-chemical nitrobenzene mixture. They used both individual chemical dose-response parameters and known mechanistic data to group chemicals. Based on this approach, they articulated three groups: 11 nitrobenzenes operating primarily through narcosis, two mononitrobenzenes acting through a redox-cycling mechanism, and a single chemical acting through an antimitotic pathway. Dose addition was used to calculate the expected joint effects from each of the two groups with more than one chemical. These two groups were then combined with the single chemical using independent action (Fig. 9.7).

Since its introduction, integrated addition has been used by various research groups to provide an additional point of comparison together with independent action and dose addition (Ra et al. 2006; Flippin et al. 2009). There have also been attempts to improve models based on integrated addition using more sophisticated statistical procedures. Mwense et al. (2004, 2006) used advanced approaches such as molecular modeling to derive chemical descriptors and fuzzy set theory to assign

chemicals to similarity groups. Qin et al. (2011, 2015) employed multiple linear regression techniques to combine predictions based on independent action and dose addition.

9.6.2 Challenges of Integrated Addition

The greatest challenge to applying integrated addition is a lack of information on the mechanisms of action or adverse outcome pathways associated with individual chemicals. Additionally, chemicals can induce more than one adverse outcome pathway (Chap. 7) and can activate different adverse outcomes depending on the species. Advances in model development address the need for knowing a priori the adverse outcome pathways associated with each individual chemical by relying on physicochemical properties and data from individual dose-response curves. These approaches, however, often require advanced statistical and toxicological understanding or access to larger datasets that may not be widely available.

Inclusion of both dose addition and independent action within a single approach necessarily includes all of the challenges associated with application of those models separately. Integrated addition does not specify a particular method for calculating predictions under dose addition. Thus, any of the dose-addition methods described above could be used in the integrated-addition framework; however, because the independent-action part of the framework requires probabilities or percentages, all of the issues associated with converting response data discussed previously also apply here.

9.7 Conclusions and Recommendations

This chapter has described the long history of using simple mathematical models to better understand the toxicity of mixtures. Over the course of the last 90 years, a lot of progress has been made in developing these tools. Despite the many overlapping approaches and the often confusing terminology, some basic principles have emerged.

- Simple mathematical models to predict mixture toxicity from individual chemical data provide a useful tool for exploring the joint action of chemicals. Most of the underlying toxicological concepts involve similarity or independence. In many cases, responses predicted using dose addition and/or independent action provide close approximations of observed mixture responses.
- Despite the many variations of dose addition available, the general concept remains intact, with specific changes resulting in potentially wider application (e.g., modification to accommodate partial agonists) without substantially changing the underlying null hypothesis.

- Deviations of observed mixture responses from predicted responses can be due to a number of factors including that the prediction model is too simple (it lacks biological complexity) or there are toxicological interactions between chemicals that are not captured by the models (e.g., greater than or less than dose-additive interactions). Significant deviations could signal that follow-up mechanistic studies are required to better understand potential interactions. Further work comparing biological models (e.g., toxicokinetic models) with these simple additivity models will be important in developing plausible interaction models as well as extensions of additivity models to reflect dose dependence or mixing ratio dependence.
- When biological evidence for similarity or for independence is weak, including more than one model (e.g., dose addition and independent action or effect summation) to compare observed and predicted responses is recommended. Since adverse outcome pathways associated with chemicals are often unknown or incomplete, including multiple models can better frame the potential range of predicted mixture responses without requiring a mechanism-based argument for application of a specific model. This range of predicted responses is not the expected range of true responses, but it characterizes responses under the null models most likely to be used.
- Much of the toxicology work on mixtures has focused on low numbers of chemicals (binary and ternary combinations), with less work addressing chemical mixtures containing 10–20 constituents. Work with higher-order mixtures will be important in determining the limitations of the models described here.

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