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The Search for Synergy: A Critical Review from a Response Surface Perspective*

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I. Introduction

The search for synergy has followed many tortuous paths during the past 100 years, and especially during the last 50 years. Claims of synergism for the effects, both therapeutic and toxic, of combinations of chemicals are ubiquitous in the broad field of Biomedicine. Over 20,000 articles in the biomedical literature from 1981 to 1987 included "synergism" as a key word (Greco and Lawrence, 1988). Travelers on the search for synergy have included scientists from the disciplines of Pharmacology, Toxicology, Statistics, Mathematics, Epidemiology, Entomology, Weed Science, and others. Travelers have independently found the same trails, paths have crossed, bitter fights have ensued, and alliances have been made. The challenge of assessing the nature and intensity of agent interaction is universal and is especially critical in the chemotherapy of both infectious diseases and cancer. In the mature field of anticancer chemotherapy, with minor exceptions, combination chemotherapy is required to cure all drug-sensitive cancers (DeVita, 1989). For the nascent field of Antiviral Chemotherapy, combination chemotherapy is of great research interest because of its great clinical potential (Schinazi, 1991). Our review should aid investigators in understanding the various rival approaches to the assessment of drug interaction and assist them in choosing appropriate approaches.

We will make no attempt to offer advice on the use of a discovery of synergy. The interpretation of the impact of both qualitative and quantitative measures of agent interaction is dependent upon the field of application. At the very least, an agent combination that displays moderate to extreme synergy can be labeled as interesting and deserving of further study. (Inventors may use proof of synergy as support for the characteristic of "unobviousness," which will assist them in receiving a patent for a combination device or formulation with the United States Patent Office.)

There have been many previous reviews of this controversial subject of agent interaction assessment. These critiques are summarized in the next section. However, our review is unique in several ways. First, our bias is toward the use of response surface concentration-effect models to aid in the design of experiments, to use for fitting data and estimating parameters, and to help in visualizing the results with graphs. In fact, because a major strength of response surface approaches is that they can help to explain the similarities and differences among other approaches, the entire review is from a response surface perspective. [Response surface methodology is composed of a group of statistical techniques, including techniques for experimental design, statistical analyses, empirical model building, and model use (Box and Draper, 1987). A response surface is a mathematical equation, or the graph of the equation, that relates a dependent variable, such as drug effect, to inputs such as drug concentrations.] Second, two common data sets, one with continuous responses and one with discrete success/failure responses, are used to compare 13 specific rival approaches for continuous data, and three rival approaches for binary success/failure data, respectively. Third, many detailed criticisms of many approaches are included in our review; these criticisms have not appeared elsewhere.

It should be noted that the goal of this review is to underscore the similarities, differences, strengths, and weaknesses of many approaches, but not to provide a complete recipe for the application of each approach. Readers who need the minute details of the various approaches should refer to the original articles. A good compendium of recipes for many of the approaches included in this review is the fourth chapter of a book by Calabrese (1991). It should also be noted that many of the approaches were originally written as guidelines, not detailed algorithms. Therefore, our specific implementations of several of the methods may have differences from the approaches actually intended by the original authors.

There is no uniform agreement on the definitions of agent interaction terms. Sources for extensive discussions of rival nomenclature include the following: Berenbaum (1989); Calabrese (1991); Copenhaver et al. (1987); Finney (1952, 1971); Gessner (1988); Hewlett and Plackett (1979); Loewe (1953); Kodell and Pounds (1985; 1991); Valeriote and Lin (1975); Unkelbach and Wolf (1984); and Wampler et al. (1992). It is our view that many of the naming schemes are unnecessarily complex. We will use a simple scheme that was the consensus of six scientists who debated concepts and terminology for agent interaction at the Fifth International Conference on the Combined Effects of Environmental Factors in Sarriselkä, Finnish Lapland, September 6 to 10, 1992 (Greco et al., 1992). The six scientists, from the fields of Pharmacology, Toxicology and Biometry, comprised a good representative sample of advocates of diametrically opposing views on many issues.

Table 1 lists the consensus terminology for the joint action of two agents, the major part of the so-called Saariselkä agreement. The foundation for this set of terms includes two empirical models for "no interaction" for the situation in which each agent is effective alone. (Even though the term "interaction" has a mechanistic connotation when applied to agent combinations, it will be used throughout this article in a purely empirical sense. Also, the less-mechanistic term, "combined-action" will be often substituted for "interaction" when

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† Abbreviations: 3-D, three-dimensional; 2-D, two-dimensional; Eq., equation; vs., versus; see table 2 for mathematical/statistical abbreviations.

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TABLE 1
Consensus terminology for two-agent combined-action concepts

	Both agents effective individually; Eq. 6 is the reference model	Both agents effective individually; Eq. 11 or 14 is the reference model	Only one agent effective individually	Neither agent effective individually
Combination effect greater than predicted	Loewe synergism	Bliss synergism	synergism	coalism
Combination effect equal to prediction from reference model	Loewe additivity	Bliss independence	inertism	inertism
Combination effect less than predicted	Loewe antagonism	Bliss antagonism	antagonism	

feasible.) The mathematical details of these two models are described in Section III, and the debate over which of these is the best null reference model is the subject of Section IV. The first model is that of Loewe additivity (Loewe and Muischnek, 1926), which is based on the idea that, by definition, an agent cannot interact with itself. In other words, in the sham experiment in which an agent is combined with itself, the result will be Loewe additivity. The second model is Bliss independence (Bliss, 1939), which is based on the idea of probabilistic independence; i.e., two agents act in such a manner that neither one interferes with the other, but each contributes to a common result. The cases in which the observed effects are more or less than predicted by Loewe additivity or Bliss independence are Loewe synergism, Loewe antagonism, Bliss synergism, and Bliss antagonism, respectively. The use of the names Loewe and Bliss as adjectives emphasizes the historical origin of the specific models and deemphasizes the mechanistic connotation of the terms additivity and independence. Both Loewe additivity and Bliss independence are included as reference models, because each has some logical basis, and especially because each has its own coterie of staunch advocates who have successfully defended their preferred model against repeated vicious attacks (see Section IV). As shown in table 1, when only one agent in a pair is effective alone, inertism is used for "no interaction," synergism (without a leading adjective) for an increased effect caused by the second agent, and antagonism for the opposite case. Alternate common terms for the latter two cases are potentiation and inhibition. When neither drug is effective alone, an ineffective combination is a case of inertism, whereas an effective combination is termed coalism.

For the cases in which more than two agents are present in a combination, it may not always be fruitful to assign special names to the higher order interactions. It may be better to just quantitatively describe the results of a three-agent or more complex interaction than to pin a label on the combined-action. However, in some fields, such as Environmental Toxicology, it may be useful to assign a descriptive name to a complex mixture of chemicals at specific concentrations. Then, six of the abovementioned terms have clear, useful extensions to higher order interactions: Loewe additivity, Loewe synergism, Loewe antagonism, Bliss independence, Bliss synergism, and Bliss antagonism. Note also that all ten terms are defined so that as the concentration or intensity of the agent(s) increases, the pharmacological effect monotonically increases. This is why the lower right-hand cell of table 1 is missing; a pharmacological effect less than zero is not defined. However, because in the field of chemotherapy it is common for increased concentrations of drugs to decrease the survival or growth of infectious agents or of tumor cells, most of the concentration-effect (dose-response) equations and curves in this review will assume a monotonically decreasing observed effect (response), such as virus titer. The dependent response variable will be labeled as effect, % effect, % survival, or % control in most graphs and will decrease with increasing drug concentration. In contrast, ID_X values such as ID_{25} will refer to the concentration of drug resulting in $X\%$ of pharmacological effect (e.g., 25% inhibition, leaving 75% of control survival). The above definitions and conventions will become clearer in later sections with the introduction of defining mathematical equations.

The emphasis of this review will be on approaches to assess combinations of agents that yield an unexpectedly enhanced pharmacological effect. Loewe additivity and Bliss independence will be used as references to give meaning to claims of Loewe synergism and Bliss synergism, respectively. Loewe antagonism will be only briefly discussed, as will synergism, antagonism, and coalism. Most concentration-effect models and curves in this review will be monotonic. Therapeutic synergy in in vivo and in clinical systems, which involves a mixture of efficacy and toxicity, and which often involves nonmonotonic concentration-effect curves for each agent individually and for the combination, will not be discussed.

The preceding discussion referred to global properties of agent combinations; i.e., it was implied that a particular type of named interaction, such as Loewe synergism, appropriately described the entire 3-D[†] concentration-effect surface. Some agent combinations may demonstrate different types of interaction at different local regions of the concentration-effect surface. When this occurs, the interaction terms in table 1 can be used to describe well defined regions. However, it is important to differentiate true mosaics of different interaction types from random statistical variation and/or artifacts caused by faulty data analysis methods. Unfortunately, rigorous methods to identify true mosaics are not yet available.

II. Review of Reviews

We have divided reviews on the subject of synergy into three classes: (*a*) whole books, some of which include new methodology, and some of which do not; (*b*) book chapters and journal articles entirely dedicated to review; and (*c*) book chapters and articles with noteworthy introductions and discussions of combined-action assessment, but which also include new specific methodology development or data analyses. Books include: Brunden et al. (1988); Calabrese (1991); Carter et al. (1983); Chou and Rideout (1991); National Research Council (1988); Pöch (1993); and Vollmar and Unkelbach (1985). Book chapters and articles dedicated to a review of the field include: Berenbaum (1977, 1981, 1988, 1989); Copenhaver et al. (1987); Finney (1952, 1971); Gessner (1988); Hewlett and Plackett (1979); Jackson (1991); Kodell and Pounds (1991); Lam et al. (1991); Loewe (1953, 1957); Rideout and Chou (1991); and Unkelbach and Wolf (1984). Book chapters and articles that include significant reviews of various approaches, but which also include either new methodology development and/or analyses of new data include: Chou and Talalay (1983, 1984); Gennings et al. (1990); Greco (1989); Greco and Dembinski (1992); Hall and Duncan (1988); Kodell and Pounds (1985); Prichard and Shipman (1990); Sühnel (1990); Syracuse and Greco (1986); Tallarida (1992); and Machado and Robinson (1994).

Although not exhaustive, this list includes a comprehensive, redundant account of the interaction assessment literature. This list includes critical and noncritical reviews of history, philosophy, semantics, approaches advocated by statisticians, and approaches advocated by pharmacologists. Most of the reviews are biased toward the respective authors' point of view, and many of the reviews harshly criticize the work of rival groups. Our review is no exception. A subset of these reviews, which along with our own, will provide a comprehensive, but not overly redundant view of the field include: chapters 1 to 4 of Calabrese (1991), which provide a relatively noncritical recipe-like description of concepts, terminology, and assessment approaches, including many disagreements with our review; chapters 1 to 2 of Chou and Rideout (1991), which also provide a contrasting view to our review on many issues; Copenhaver et al. (1987), which accents the approaches developed by statisticians; Berenbaum (1981, 1988, 1989), which critically review the approaches developed by pharmacologists; Gessner (1988), which examines approaches developed both by statisticians and pharmacologists; and Kodell and Pounds (1991), which may be the best source for a rigorous comparison of rival concepts and nomenclature.

III. General Overview of Methods from a Response Surface Perspective

Figure 1 is a schematic diagram of a general approach to the assessment of the nature and intensity of drug interactions. This scheme includes all of the approaches examined in later sections. This is because, in essence, figure 1 describes the scientific method. A formal statistical response surface way of thinking underlies all of this section. With such an orientation, the similarities and differences among rival approaches for the assessment of drug interactions, both mathematically rigorous ones and not-so-rigorous ones, can be readily explained.

Step 1 is to choose a good concentration-effect (dose-response) structural model for each agent when applied individually. A common choices is the Hill model (Hill, 1910), which is also known as the logistic model (Waud and Parker, 1971; Waud et al., 1978). The Sigmoid-Emax model (Holford and Sheiner, 1981), is equivalent to a nonlinear form of the median-effect model (Chou and Talalay, 1981, 1984). However, the equivalence of the median-effect and Hill models is disputed by Chou (1991). The Hill model is shown in figure 2 and as Eq. 1 for an inhibitory drug. Symbol definitions are listed in table 2.

$$E = \frac{Emax\left(\frac{D}{IC_{50}}\right)^m}{1+\left(\frac{D}{IC_{50}}\right)^m} \qquad [1]$$

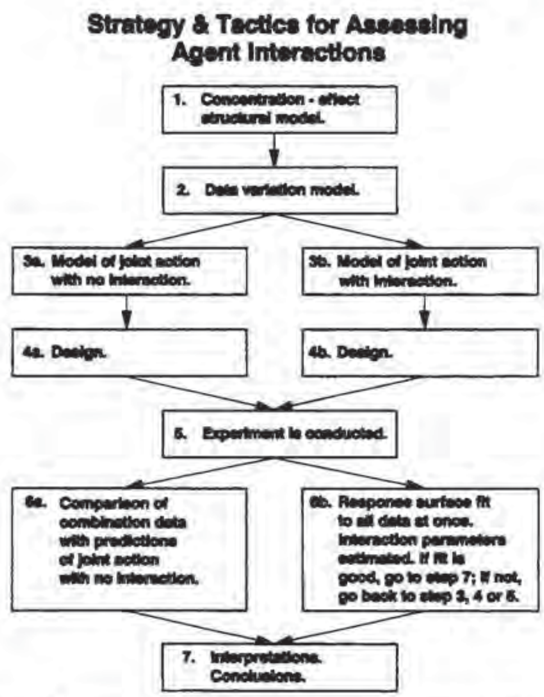


FIG. 1. Schematic diagram of a general approach to the assessment of the nature and intensity of agent interactions, which includes all specific approaches.

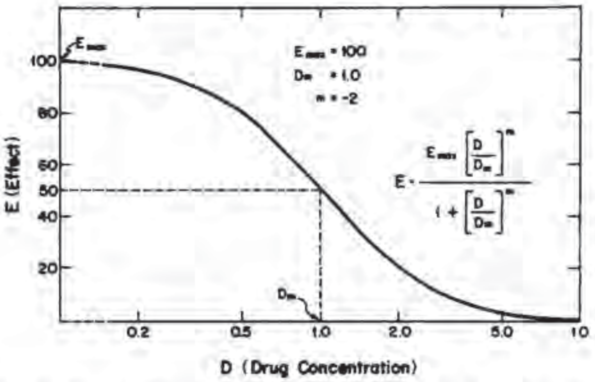


FIG. 2. Graph of the Hill (1910) model, which is also referred to as the Sigmoid-Emax model (e.g., Holford and Sheiner, 1981), and which is also a nonlinear form of the median-effect equation (Chou and Talalay, 1984).

In Eq. 1, E is the measured effect (response), such as the virus titer remaining in a culture vessel after drug exposure; D is concentration of drug; $Emax$ is the full range of response that can be affected by the drug; Dm or IC_{50} is the median effective dose (or concentration) of drug (or ID_{50}, ED_{50}, LD_{50}, etc.); and m is a slope parameter. When m has a negative sign, the curve falls with increasing drug concentration; when m is positive, the curve rises with increasing drug concentration. The concentration-effect curve in figure 2 can be thought of as an ideal curve formed by data with no discernible variation, or as the true curve known only to God or to Mother Nature, or as the average curve formed by an infinite number of data points at each of an infinite number of evenly spaced concentrations. Equations 2 to 4 are additional candidate structural models for single agents.

$$E = \frac{Econ\left(\frac{D}{IC_{50}}\right)^m}{1 + \left(\frac{D}{IC_{50}}\right)^m} \qquad [2]$$

$$E = \frac{(Econ - B)\left(\frac{D}{IC_{50}}\right)^m}{1 + \left(\frac{D}{IC_{50}}\right)^m} + B \qquad [3]$$

$$E = Econ\ \exp(aD) = Econ\ \exp\left(\frac{D\ln\left(\frac{1}{2}\right)}{IC_{50}}\right) \qquad [4]$$

In Eqs. 2 and 3, the parameter $Econ$ is the control effect (or response when no inhibitory drug is applied). When there is no B (background response observed at infinite drug concentration), then $Econ$ is equivalent to $Emax$, as in Eq. 2. However, when there is a finite B, then $Econ = Emax + B$. Eq. 4 is the exponential concentration-effect model, which can also be parameterized with an IC_{50}.

Because real experiments rarely generate data that fall on the ideal curve, Step 2 in figure 1 is to choose an appropriate data variation model. Model candidates include the normal distribution for continuous data, such as found in growth assays in which the absorbance of a dye bound to cells is the measured signal; the binomial distribution (Larson, 1982) for proportions of failures or successes, such as in acute toxicology experiments; and the Poisson distribution for low numbers of counts, such as in clonogenic assays. A composite model is formed from one structural model plus one data variation model and eventually used for fitting to real experimental data. This concept, called generalized nonlinear modeling (McCullagh and Nelder, 1989) is illustrated in figure 3, with the Hill model as the structural model, and the normal, binomial, and Poisson distributions (respectively from left to right) as the random models. (Note that only one random component is usually assumed for a particular data set. Graphs of three random components are pictured in figure 3 to illustrate the universal nature of the approach. The lower equation in the figure is a variant of the Hill model, and the upper one is for the binomial distribution. These equations will be described in detail in Section VI.)

In Step 3, most approaches can be categorized into one of two main strategies. In Step 3a, a structural model is derived for joint action of two or more agents with the assumption of "no interaction" (Loewe additivity, Bliss independence, or another null reference model). Then, after the experiment is designed and conducted, data from the combination of agents is compared with predictions of joint action from a null reference combined-action model. This comparison can be made with formal statistical rejections of null hypotheses, or by less formal methods. In contrast, in Step 3b, a structural model is derived for joint action that includes interaction terms. Then, after the experiment is designed and conducted, the full combined-action model is fit to all of the data at once, and interaction parameters are estimated. Both the left-hand and right-hand strategies end in a set of guidelines for making conclusions.

Examples of approaches that use the left-hand strategy include: the classical isobologram approach (Loewe and Muischnek, 1926); the fractional product method of Webb (1963); the method of Valeriote and Lin (1975); the method of Drewinko (1976); the method of Steel and Peckham (1979); the method of Gessner (1974); the methods of Berenbaum (1977, 1985); the median-effect method (Chou and Talalay, 1981, 1984); the method of Prichard and Shipman (1990); and the method of Laska et al. (1994). Examples of approaches that use the right-hand strategy include the universal response surface approach (Greco et al., 1990; Greco and Lawrence, 1988; Greco, 1989; Greco and Tung, 1991; Syracuse and Greco, 1986); the response surface approaches of Carter's group

TABLE 2
Mathematical / statistical symbol definitions

Symbol	Definition
E	Measured effect (or response), in this review, usually a measure of survival
Y	Transformed response variable, continuous or discrete
y	A particular value of Y
$P(.)$	Probability that the function in parenthesis is true
μ	Mean or expected value of a transformed response
k	Number of successes in a binomial trial
n	Number of attempts in a binomial trial
D, [drug], D_1, [drug 1], D_2, [drug 2]	Concentration (or dose) of drug, drug 1, drug 2
I, I_1, I_2	Inhibitor concentrations for an inhibitor, inhibitor 1, inhibitor 2
$Econ$	Control effect (or response)
$Emax$	Maximum effect (response), is equal to $Econ$ for an inhibitory drug in the absence of a background, B
B	Background effect (response) observed at infinite concentration for an inhibitory drug
fa	Fraction of effect affected
fu	Fraction of effect unaffected
fi	Fraction enzyme velocity inhibited
$IC_{50}, I_{50}, IC_{50,1}, IC_{50,2}$	Concentration (or dose) of drug resulting in 50% inhibition of $Emax$, of drug 1, of drug 2
$Dm, Dm_1, Dm_2\ Dm_{12}$	Median effective dose (or concentration) of drug, of drug 1, of drug 2, of a combination of drugs 1 and 2 in a constant ratio (equivalent to IC_{50})
$ID_X, D_X, IC_X, ID_{X,1}, D_{X_1}, ID_{X,2}, Dx_2, Dx_{12}$	Concentration (or dose) of drug resulting in X% inhibition of $Emax$, of drug 1, of drug 2, or a combination of drugs 1 and 2 in a constant ratio
X	% inhibition
m, m_1, m_2, m_{12}	Slope parameter, for drug 1, for drug 2, for a combination of drugs 1 and 2 in a constant ratio
α	Synergism-antagonism interaction parameter
a, b	Empirical parameters for exponential concentration-effect model
PC_1, PC_2, bp_1, bp_2	Interaction parameters of model 29
η	Interaction parameter of model 30
$\beta_0, \beta_1, \beta_2, \beta_{12}$	Empirical parameters for probit and logistic models
I	Interaction index of Berenbaum (1977)
CI	Combination index of Chou and Talalay (1984)
R	Ratio of D_1 to D_2

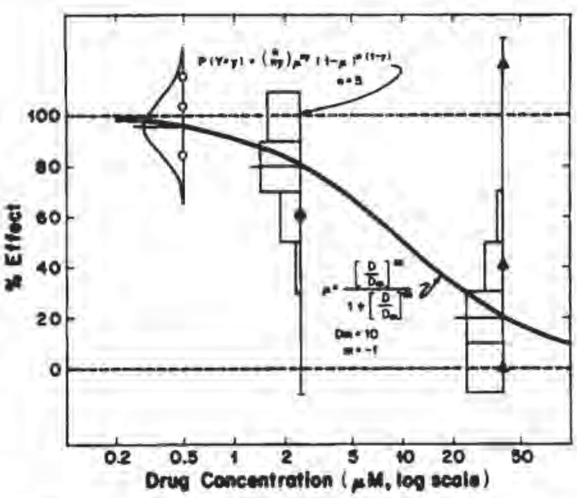


FIG. 3. General scheme for the dissection of a generalized nonlinear model into random and structural components for a concentration-effect curve for a single drug.

(Carter et al., 1983, 1986, 1988; Gennings et al., 1990); the response surface approach of Weinstein et al. (1990); the generalized linear model approach of Lam et al. (1991); and the response surface approach of Machado and Robinson (1994). The method proposed by Sühnel (1990) has elements of both the left-hand and right-hand strategies.

Although most, and possibly all, approaches for assessing agent combinations may fall under the scheme presented in figure 1, the different approaches differ from each other in many respects. The approaches developed by pharmacologists usually stress structural models, e.g., the median-effect approach (Chou and Talalay, 1984), whereas the approaches developed by statisticians usually stress data variation models, e.g., the approaches of Finney based on probit analysis (Finney, 1952). There are differences in the definitions of key terms, especially that of "synergism." Some approaches only yield a qualitative conclusion (e.g., Loewe synergism, Loewe antagonism, or Loewe additivity), such as the classical isobologram approach, whereas others also provide a quantitative measure of the intensity of the interaction, such as the universal response surface approach. There are differences in the degree of mathematical and statistical rigor, i.e., some approaches are performed entirely by hand (e.g., the classical isobologram approach), whereas others require a computer (e.g., universal response surface approach). Some approaches use parametric models (e.g., Greco et al., 1990), whereas others emphasize nonparametric models (e.g., Sühnel, 1990; Kelly and Rice, 1990). The suggested designs for experiments differ widely among the different approaches. It is therefore not surprising that it is possible to generate widely differing conclusions on the nature of a specific agent interaction when applying different methods to the same data set. This will be illustrated dramatically in Sections V and VI.

We are highly biased in our view that the right-hand strategy in figure 1 for assessing agent interactions is superior to the left-hand strategy when used for the cases in which an appropriate response surface model can be found to adequately model the biological system of interest. However, for preliminary data analyses for all systems, for the final data analyses of complex systems, and for cases in which the data is meager, the left-hand approaches are often very useful.

The derivation of Eq. 5, the flagship equation for two-agent combined-action developed by our group, is provided in detail in Greco et al. (1990). Although we do not put forward Eq. 5 as *the* model of two-agent combined-action, it is *a* model of two-agent combined-action that has proved to be very useful for both practical applications (Greco et al., 1990; Greco and Dembinski, 1992; Gaumont et al., 1992; Guimarãs et al., 1994) and methodology development (Syracuse and Greco, 1986; Greco and Lawrence, 1988; Greco, 1989; Greco and Tung, 1991; Khinkis and Greco, 1993; Khinkis and Greco, 1994; Greco et al., 1994). Eq. 5 will be used throughout this review to illustrate concepts of combined-action and to assist in the comparison of rival data analysis approaches. Eq. 5 was derived with an adaptation of an approach suggested by Berenbaum (1985), with the assumption of Eq. 2 as the appropriate model for each agent alone. The interaction parameter is α.

$$1 = \frac{D_1}{IC_{50,1}\left(\frac{E}{Econ - E}\right)^{1/m_1}} + \frac{D_2}{IC_{50,2}\left(\frac{E}{Econ - E}\right)^{1/m_2}} + \frac{\alpha D_1 D_2}{IC_{50,1} IC_{50,2}\left(\frac{E}{Econ - E}\right)^{(1/2m_1 + 1/2m_2)}} \quad [5]$$

Eq. 5 allows the slopes of the concentration-effect curves for the two drugs to be unequal. It is this key feature that distinguishes Eq. 5 from many other response surface models used by others to describe agent interactions (e.g., Carter et al., 1988). (This point is expanded in Section VI. B.2.). Because Eq. 5 is in unclosed form (the dependent variable, E, cannot be isolated on the left-hand side of the equation), a one-dimensional bisection root finder (a computer numerical procedure explained, e.g., by Thisted, 1988) is used to calculate E for simulations. Eq. 5 was not derived from biological theory, rather it is an empirical equation that often matches the shape of real data (e.g., Gaumont et al., 1992; Greco et al., 1990; Greco and Dembinski, 1992; Greco and Lawrence, 1988). However, as shown below, it is consistent with Eq. 6, the equation for Loewe additivity (Loewe and Muischnek, 1926), which is the basis of many interaction assessment approaches.

$$1 = \frac{D_1}{ID_{X,1}} + \frac{D_2}{ID_{X,2}} \quad [6]$$

For an inhibitory drug, Eq. 6 refers to a particular $X\%$ (percent inhibition level), e.g., 58% inhibition. $ID_{X,1}$, $ID_{X,2}$ are the concentrations of drugs to result in $X\%$ inhibition for each respective drug alone, and D_1, D_2 are concentrations of each drug in the mixture that yield $X\%$ inhibition. When the right-hand side of Eq. 6 [equal to the Interaction index, I, of Berenbaum (1977) or to the combination index, CI, for the mutually exclusive case of Chou and Talalay (1984)] is less than 1, then Loewe synergism is indicated, and when the right-hand side is greater than 1, Loewe antagonism is indicated. When Eq. 2 is an appropriate concentration-effect model for each drug alone, then Eq. 7, which is a rearrangement of Eq. 2 [similar to a rearrangement of the median-effect equation from Chou and Talalay (1984)], relates the ID_X value for any $X\%$ inhibition to the observed response level, E, and the parameters, $Econ$, IC_{50}, and m.

$$ID_X = IC_{50}\left(\frac{E}{Econ - E}\right)^{1/m} \quad [7]$$

Note that the right-hand expression of Eq. 7 is the same as the denominators of the first two right-hand terms of Eq. 5. Therefore, the first two right-hand terms of Eqs. 5 and 6 are equivalent. It follows that Eq. 8 defines I [or CI for the mutually exclusive case of Chou and Talalay (1984)] for two-drug combinations whose individual components have concentration-effect curves that follow Eq. 2.

$$I = CI = \frac{D_1}{IC_{50,1}\left(\frac{E}{Econ - E}\right)^{1/m_1}} + \frac{D_2}{IC_{50,2}\left(\frac{E}{Econ - E}\right)^{1/m_2}} = 1 - \frac{\alpha D_1 D_2}{IC_{50,1} IC_{50,2}\left(\frac{E}{Econ - E}\right)^{(1/2m_1 + 1/2m_2)}} \quad [8]$$

Therefore, based upon the interaction index, I—when α is positive, Loewe synergism is indicated, when α is negative, Loewe antagonism is indicated, and when α is 0, Loewe additivity is indicated. The magnitude of α indicates the intensity of the interaction. Thus, although Eq. 5 is not *the* model for Loewe synergism (or Loewe antagonism), it is *a* model for Loewe synergism (or Loewe antagonism) that is consistent with the more general Loewe additivity model, Eq. 6.

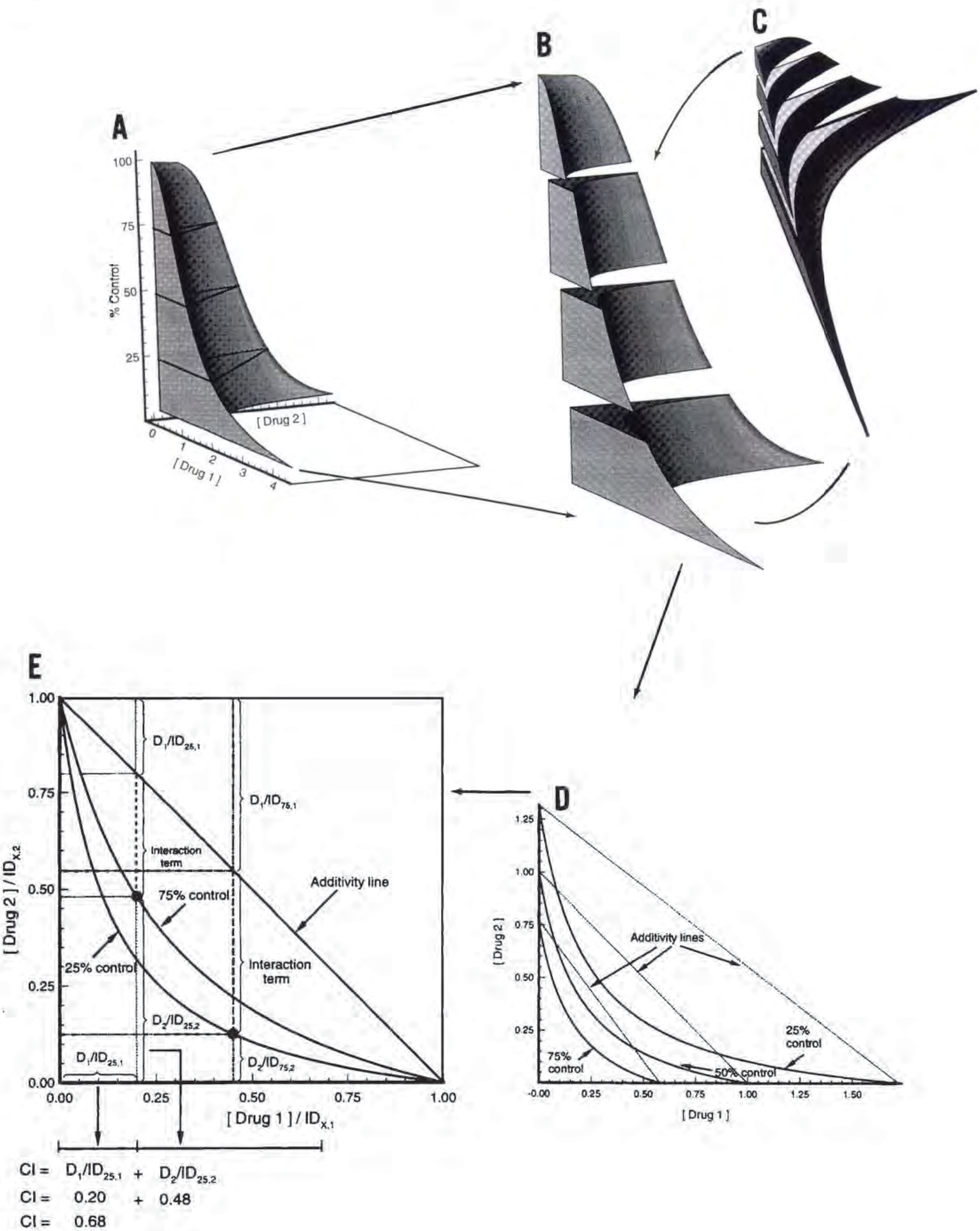

A
B
C
D
E
100
75
50
25
% Control
0
1
2
3
4
[Drug 1]
[Drug 2]
1.00
0.75
0.50
0.25
0.00
[Drug 2] / $ID_{X,2}$
[Drug 1] / $ID_{X,1}$
$D_1/ID_{25,1}$
$D_1/ID_{75,1}$
Interaction term
Additivity line
75% control
25% control
$D_2/ID_{25,2}$
$D_2/ID_{75,2}$
CI = $D_1/ID_{25,1}$ + $D_2/ID_{25,2}$
CI = 0.20 + 0.48
CI = 0.68
1.25
-0.00
1.50
Additivity lines
25% control
75% control
50% control

FIG. 4.

We now use the concept that Eq. 5 generates a Loewe synergistic response surface at all effect levels, and we present several 3-D and 2-D graphical representations of Eq. 5 to help to show the similarities and differences among the various approaches to the assessment of Loewe synergism.

Figure 4 shows the relationship between a 3-D response surface of Loewe synergism, the construction of isobols, and the calculation of interaction indices. The 3-D surface was simulated with Eq. 5, our flagship model for agent interaction for the case in which the individual drugs follow the Hill model, Eq. 2, with unequal slope parameters. The interaction parameter, α, was made equal to 5 to demonstrate strong synergism. The other parameters used and additional technical details are listed in the figure legend. Note the scooped out nature of the Loewe synergistic surface in contrast to the three Loewe additivity bars at 75%, 50%, and 25% of control. A complete Loewe additivity surface (α = 0) would consist of straight lines running across the surface parallel to these bars at every effect level. To show the 3-D origin of 2-D isobols, the surface is cut and separated at the 25%, 50%, and 75% effect levels and rotated so that the viewer sees the surface from the top. The isobols in panel (D) are not symmetric because of the different slope parameters for drug 1 (m = -1) and drug 2 (m = -2). However, as seen in panel (E), normalizing the drug concentrations by the respective ID_X values (from Eq. 7) makes the isobols symmetric. In addition, the normalization reverses the order of the isobols and makes the Loewe additivity lines lie on top of each other for all effect levels. Panel (E) shows the geometrical relationships among normalized isobols, interaction (or combination) indices, and response surface equations. One specific CI calculation is given for one specific point on the 25% pharmacological effect (75% control) isobol. The calculated CI is 0.68, indicating Loewe synergism. Vertical lines, made up of three different line patterns, run through the two data points. The three segments of each line correspond to the three right-hand parts of the response surface model, Eq. 5.

The geometrical relationships between interaction models and isobols are further examined in figure 5. Note in panel A that lines at a 45° angle in the northeast direction between the isobol and the Loewe additivity diagonal are equal to the interaction term divided by $\sqrt{2}$. In panel (B), panel (A) is redrawn with the curves removed, with many horizontal, vertical and diagonal lines drawn, and with vertices labeled. These reference lines and ubiquitous 45° triangles all aid in the interpretation of the geometry of the 25% isobol (75% control). In panel (B), the length of each thick line represents the magnitude of the interaction term. This is a general result and will be true for a large class of specific equations that follow the general interaction equation, Eq. 9.

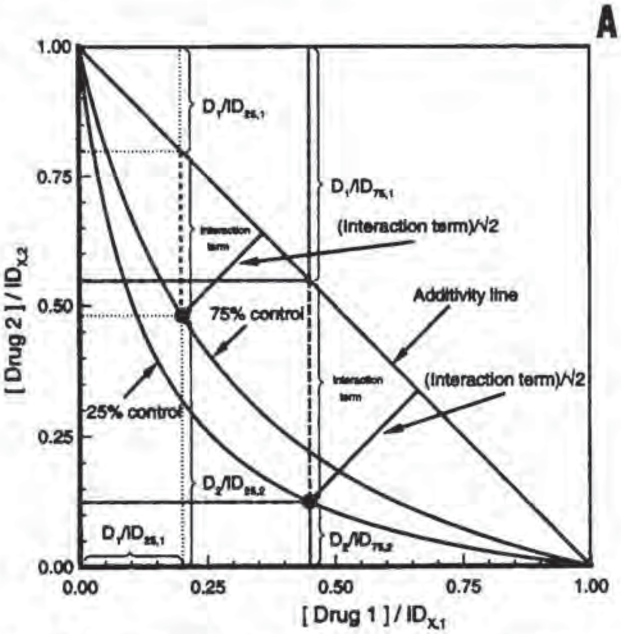
$$1 = \frac{D_1}{ID_{X,1}} + \frac{D_2}{ID_{X,2}} + f\left(\frac{D_1}{ID_{X,1}}, \frac{D_2}{ID_{X,2}}, \alpha, \mathbf{p}\right) \qquad [9]$$

Eq. 9 is a general form that is independent of the specific concentration-effect models for each drug (that may be different for each drug). Also, the interaction term may be any function of the normalized concentrations, may include any number of interaction parameters, $\boldsymbol{\alpha}$, and may include any number of additional parameters, $\mathbf{p}$. Additional specific response surface interaction models, including ones from Weinstein et al. (1990) and Machado and Robinson (1994), which are consistent with Eq. 9, are described in Section V.L.

Figure 6 shows the geometrical relationships for 50% effect isobols for Eq. 5, with various values of α listed in the figure legend. When α is positive, the isobols are to the left of the Loewe additivity diagonal (α = 0), line E; larger α values increase the bowing of the isobols, indicating more intense Loewe synergism. When α is negative, the isobols are to the right of the Loewe additivity diagonal; as α increases in absolute value, the isobols become more bowed, indicating more intense Loewe antagonism. The degree of bowing of the isobols can be quantitated as the ratio of the line segments, S = on/om (Hewlett, 1969) or by the sum of op + oq (Elion et al., 1954). The interaction parameter, α, is related to these geometrical measures (Greco et al., 1990). Eq. 10 was derived by Greco et al. (1990) and shows the relationship between α and S for the 50% effect isobols of Eq. 5.

$$\alpha = 4(S^2 - S) \qquad [10]$$

FIG. 4. Illustration of the relationship between a 3-D response surface of Loewe synergism, the construction of isobols, and the calculation of combination (interaction) indices. (*A*) A hypothetical 3-D solid shaded graph of measured effect (response, survival, or some other endpoint) expressed as a percent of control effect vs. the concentrations of drug 1 and drug 2. This graph was simulated with Eq. 5, with parameters: $Econ$ = 100, $IC_{50,1}$ = 1, $IC_{50,2}$ = 1, m_1 = -1, m_2 = -2, α = 5. The horizontal lines connecting the edges of the surface at 75%, 50%, and 25% of control are part of a Loewe additivity surface (Eq. 5, α = 0). (*B*) The surface is cut and separated at the 75%, 50%, and 25% of control levels, and the sections are pulled apart to accent the inward curved shape of the surface. (*C*) The sectioned surface is being rotated so that the viewer will be able to see the surface from the top. (*D*) A view of the surface from the top; a set of 2-D isobols at 75%, 50%, and 25% of control, along with their corresponding Loewe additivity lines. (*E*) An isobologram in which the isobols at 75% and 25% of control each have their drug concentrations normalized by their respective ID_Xvalues. This makes all of the isobols symmetrical, makes all of the Loewe additivity lines coincide, and reverses the order of the isobols. Two vertical lines, each running the full length of the Y-axis, and each comprised of three segments of different line patterns, one for the 25% isobol (75% of control) and one for the 75% isobol (25% of control) show the correspondence between the isobol diagram and Eq. 5. Each of the three segments corresponds to one of the three right-hand expressions of Eq. 5. In addition, the correspondence of the combination (or interaction) index, CI, and the isobols and Eq. 5 is illustrated.



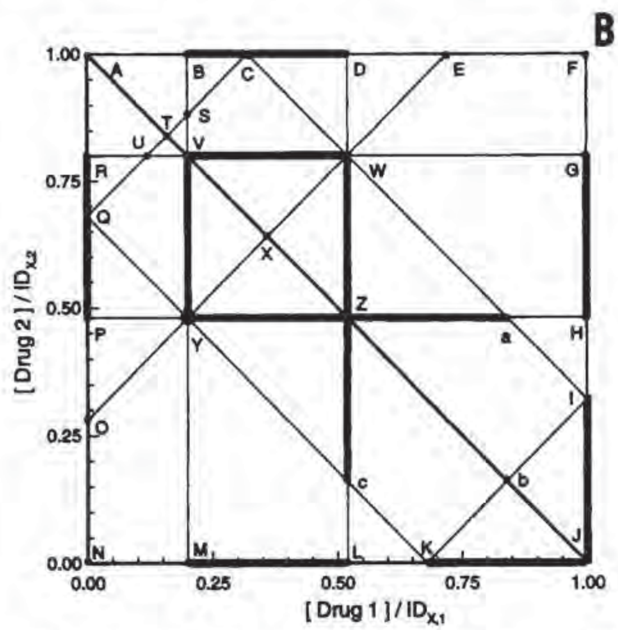


FIG. 5. Diagram to show the general correspondence between the geometry of interaction isobols and the algebraic expressions of interaction mathematical models. (*A*) An elaboration of Figure 4, Panel(*E*), which shows the correspondence between the lengths of line segments in the normalized isobologram and the value of the three right-hand expressions in Eq. 5 at 75% of control. Note that the interaction term that contains α, is the vertical distance between the curved isobol and the additivity line. (*B*) Panel (*A*) is redrawn, but only for the 25% isobol (75% of control) (with the curve removed), and many horizontal, vertical, and diagonal lines drawn and vertices labeled. The length of each thick line is equal to the value of the interaction term. This is a general correspondence, and will be true for many specific models that follow the general form of Eq. 9.

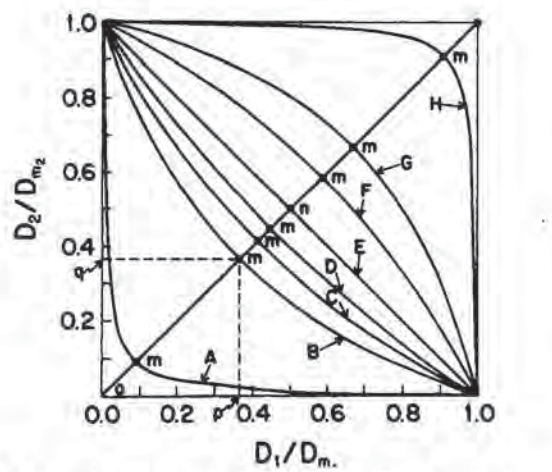


FIG. 6. Examples of isobols for the 50% effect level, simulated from equation 5, for a range of α values. For curves A through H, α was 100, 2, 1, 0.5, 0, -0.5, -0.75, and-0.99. Curves A through D represent varying degrees of Loewe synergism; curves F through H represent varying degrees of Loewe antagonism, and curve E is the straight line of Loewe additivity. The point n is the center of the straight Loewe additivity line, and points m are the centers of the other isobols. Points p and q are the abscissa and ordinate of the point m. The degree of bowing of the isobols can be quantitated as the ratio of the line segments, S = om/on (Hewlett, 1969) or by the sum of op + oq (Elion et al., 1954).

Figure 7 shows the relationship between the same 3-D response surface described in figure 5 and the concept of the *CI* vs. *fa* plot (mutually exclusive case) of the median-effect approach (Chou and Talalay, 1984). Although the exact calculations for the *CI* vs. *fa* plot suggested by Chou and Talalay (1984) will be disputed in Section V.G, we believe that the general idea has great merit. Essentially, the 3-D surface is cut lengthwise along a fixed ratio of D_1:D_2 (for example, a ratio of 1:1 in fig. 7). Then, both the Loewe synergistic ray and the predicted Loewe additivity ray are drawn on a 2-D concentration-effect graph, both rays are normalized by the ID_X values along their whole lengths, and then the normalized graph is rotated counterclockwise by 90°. The details are provided in the figure legend.

Figure 8 is another graphical sequence, using the same simulated 3-D surface as shown in figures 4, 5, and 7, created to illustrate the concept of the *CI* vs. *fa* plot. In panel (A), the Loewe synergistic surface is deleted except for one vertical, infinitely thin slice for the fixed ratio of D_1:D_2 of 1:1. The length of the short horizontal line segments at various effect levels drawn from the curve to the backplanes are the values of D_1 and D_2 used to construct the Loewe synergistic surface. Panels (B) and (C) show the unnormalized and normalized set of isobols, respectively. The solid points in these panels are the same ones as in figure 7. The sum of one vertical and one horizontal line from each point in Panel C is equal to the *CI* at that effect level. Details are provided in the figure legend.

Thus, in essence, the isobologram approach consists of making horizontal slices through a 3-D surface, and the median-effect approach (mutually exclusive case) con-

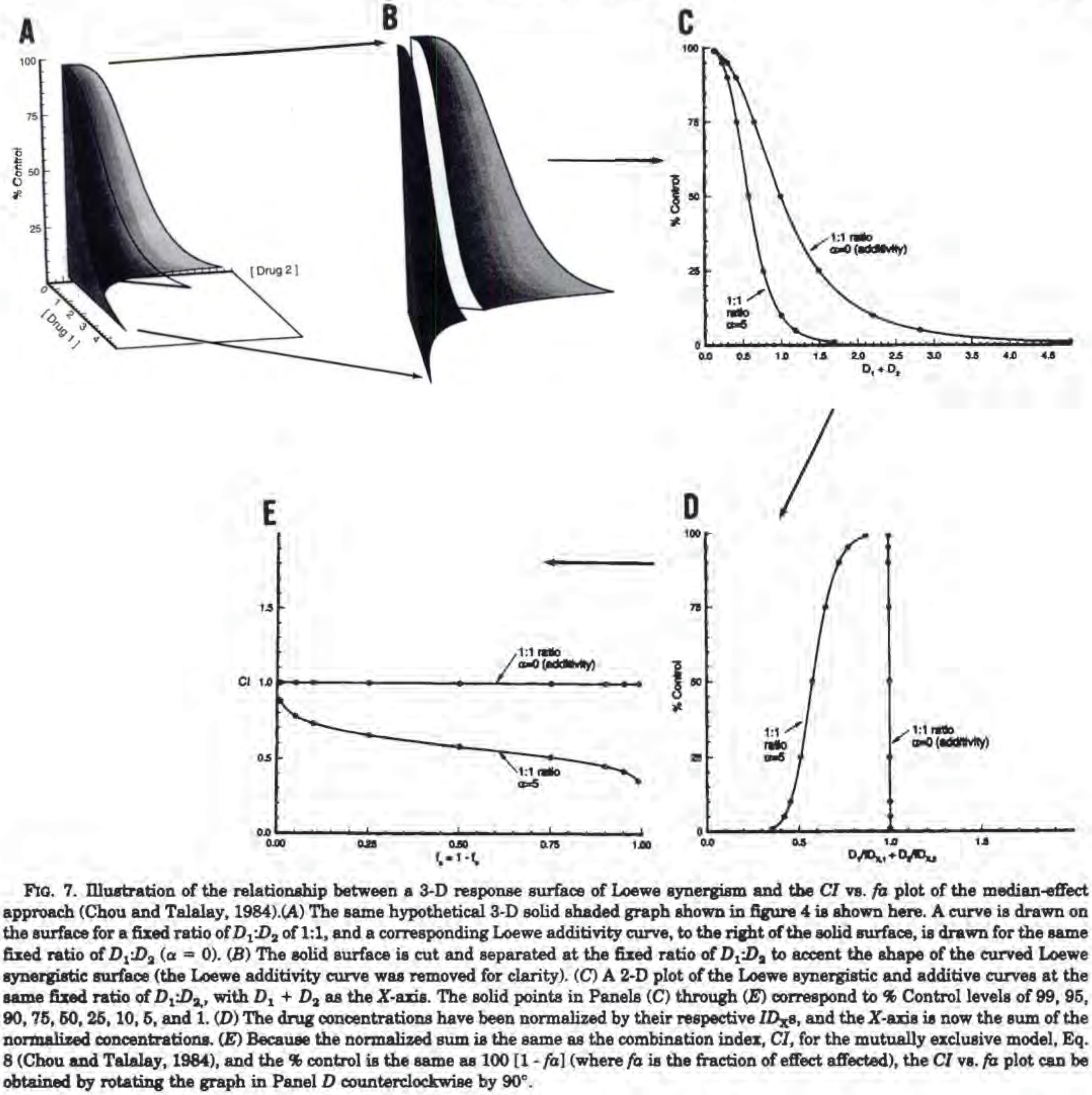


FIG. 7. Illustration of the relationship between a 3-D response surface of Loewe synergism and the *CI* vs. *fa* plot of the median-effect approach (Chou and Talalay, 1984).(*A*) The same hypothetical 3-D solid shaded graph shown in figure 4 is shown here. A curve is drawn on the surface for a fixed ratio of D_1:D_2 of 1:1, and a corresponding Loewe additivity curve, to the right of the solid surface, is drawn for the same fixed ratio of D_1:D_2 ($\alpha = 0$). (*B*) The solid surface is cut and separated at the fixed ratio of D_1:D_2 to accent the shape of the curved Loewe synergistic surface (the Loewe additivity curve was removed for clarity). (*C*) A 2-D plot of the Loewe synergistic and additive curves at the same fixed ratio of D_1:D_2, with $D_1 + D_2$ as the *X*-axis. The solid points in Panels (*C*) through (*E*) correspond to % Control levels of 99, 95, 90, 75, 50, 25, 10, 5, and 1. (*D*) The drug concentrations have been normalized by their respective ID_Xs, and the *X*-axis is now the sum of the normalized concentrations. (*E*) Because the normalized sum is the same as the combination index, *CI*, for the mutually exclusive model, Eq. 8 (Chou and Talalay, 1984), and the % control is the same as 100 [1 - *fa*] (where *fa* is the fraction of effect affected), the *CI* vs. *fa* plot can be obtained by rotating the graph in Panel *D* counterclockwise by 90°.

sists of making vertical slices through the same 3-D surface. Both approaches and their variants then include examination of the shape of the slices, with or without data transformations, and/or making some calculations to summarize the shape of the slices, usually with comparison to a Loewe additivity reference.

The difference between a Loewe synergistic surface and a Loewe additivity reference surface can also be examined in 3-D. The difference can be calculated in the horizontal or vertical directions, and plotted, with or without additional transformations. The use of difference surfaces to examine combined-actions has been introduced by Prichard and Shipman (1990) and Sühnel (1992c). The 3-D *CI* plot in figure 9 was calculated with Eq. 8 for the same simulated Loewe synergistic surface ($\alpha = 5$) shown in the previous figures. Note that *CI* starts at 1 for each drug alone, and decreases toward zero for combinations as either drug concentration increases toward infinity. Thus, for Loewe synergistic drug combinations that follow Eq. 5, there is more intense interaction, as quantified by *CI* (or *I*), at higher drug concentrations. In contrast, figure 10 [panels (A) and (C)] shows the results of plotting the vertical difference between the Loewe synergistic ($\alpha = 5$) and additivity surface. Panel (A) shows the Loewe synergistic surface with a fishnet and the Loewe additivity surface as a

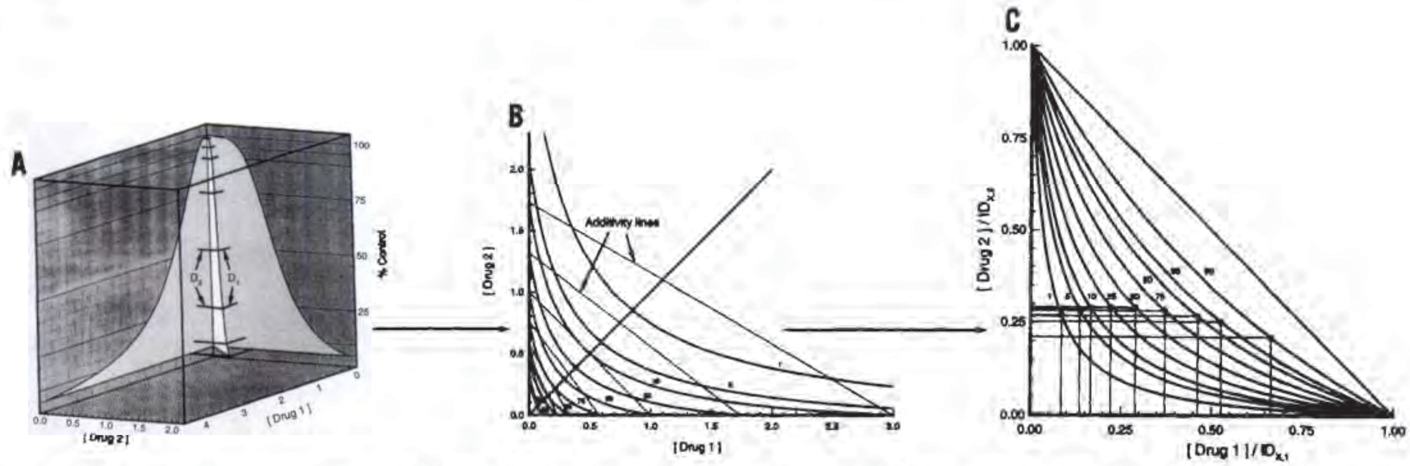


FIG. 8. An additional illustration of the relationship between a 3-D response surface of Loewe synergism and the combination index, *CI*. (*A*) For the same hypothetical surface shown in Figs. 4, 5, and 7, the concentration-effect curves for drug 1 and drug 2 alone are shown along the back walls of the figure, together with the Loewe synergistic middle curve for a fixed ratio of D_1:D_2 of 1:1. Line segments from the joint drug curve to the back walls represent the values of D_1 and D_2 used to construct the curve at % Control values of 99, 95, 90, 75, 50, 25, 10, and 5. (*B*) A view of the isobols for the surface from the top. The numbers next to the isobols indicate the % Control. The solid dots along the northeast-pointing diagonal indicate the points corresponding to the fixed ratio of D_1:D_2 of 1:1 at the indicated levels of % Control. Although not included in Panel (*B*), the line segments in Panel A would be horizontal and vertical lines from the dots to the axes. (*C*) The [Drug 1] and [Drug 2] axes are normalized by the respective ID_X values. The addition of the lengths of a horizontal plus a vertical line segment for each solid dot equals the *CI* for the respective % Control level. These points correspond to the respective points in figure 7.

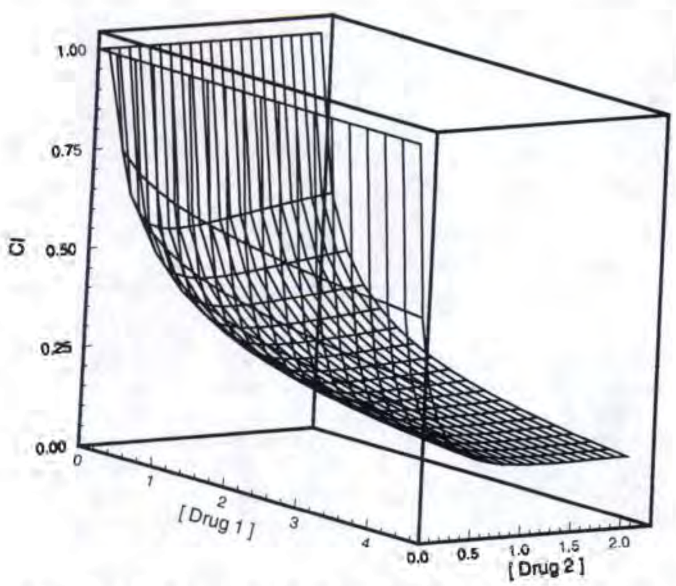


FIG. 9. A 3-D fishnet plot of the *CI* calculated from Eq. 8 for the Loewe synergistic concentration-effect surface described in Figs. 4, 5, 7, and 8.

solid sheet on top of the fishnet. Note that the difference between the two surfaces, shown in panel (C), has a peak near $D_1 = D_2 = 1$. Thus, when looking at vertical differences, the largest synergism is not at infinite drug concentrations, but rather at achievable drug concentrations near (but not exactly at) the IC_{50}'s of each drug. This critical difference in the two ways of forming differences between Loewe synergistic and Loewe additive surfaces, i.e., either in the horizontal or vertical direction, has profound implications for experimental design, as discussed in Section VIII.

In figure 10, panels (B) and (D) were constructed in an analogous manner to panels (A) and (C), except that the null reference model was that for Bliss independence, not for Loewe additivity. The general form of the Bliss independence effects equation is Eq. 11, and a specific form, which assumes that Eq. 2 is appropriate for each drug individually, is Eq. 12.

$$fu_{12} = fu_1 fu_2 \qquad [11]$$

$$E = \frac{Econ\left(\frac{D_1}{IC_{50,1}}\right)^{m_1}\left(\frac{D_2}{IC_{50,2}}\right)^{m_2}}{\left(1+\left(\frac{D_1}{IC_{50,1}}\right)^{m_1}\right)\left(1+\left(\frac{D_2}{IC_{50,2}}\right)^{m_2}\right)} \qquad [12]$$

In Eq. 11, fu_1, fu_2, and fu_{12} are the fractions of possible response for drug 1, drug 2, and the combination (e.g., % survival, %control) unaffected (Chou and Talalay, 1984). For Eqs. 2, 5, and 12, $fu = E/Econ$. Eq. 12 was used to generate the upper solid surface in panel B. Note that the difference plot in panel (D) has a central peak, but the peak is higher than the analogous one for the Loewe additivity reference in panel (C).

Which is a more appropriate reference, Loewe additivity (generally represented by Eq. 6) or Bliss independence (generally represented by Eq. 11)? Some of the approaches for interaction assessment examined in Section V use the Loewe additivity reference and others use the Bliss independence reference. This controversy is examined in detail in Section IV.

Our preferred paradigm of interaction assessment is most closely akin to the philosophical principles expressed by Berenbaum (1981, 1985, 1988, 1989), but

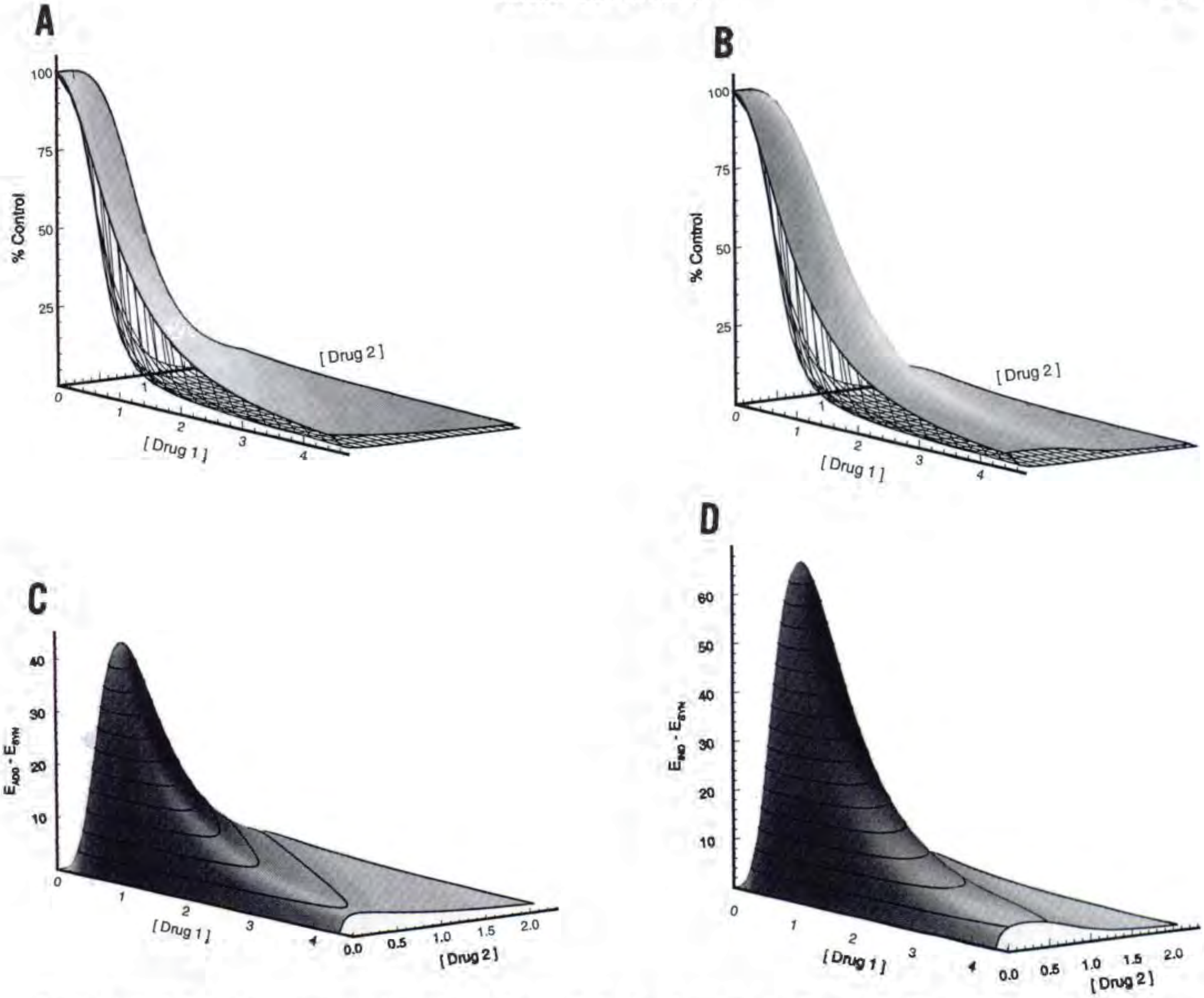


FIG. 10. (*A*) 3-D fishnet Loewe synergistic surface simulated with Eq. 5, with parameters: $Econ = 100, IC_{50,1} = 1, IC_{50,2} = 1, m_1 = -1, m_2 = -2, \alpha = 5$, the same as in Figs. 4, 5, and 7 through 9. The solid surface on the top of the fishnet is a Loewe additivity surface simulated with the same values for the first five parameters, but with $\alpha = 0$. (*B*) The 3-D fishnet Loewe synergistic surface is the same one as in Panel (*A*), but the solid top surface was simulated from the Bliss independence model, Eq. 12, with parameters: $Econ = 100, IC_{50,1} = 1, IC_{50,2} = 1, m_1 = -1, m_2 = -2$. *C*. A 3-D solid shaded graph of the difference between the Loewe additivity and Loewe synergistic surfaces in Panel (*A*). The contour lines are at five-unit intervals. (*D*) A 3-D solid shaded graph of the difference between the Bliss independence and Loewe synergistic surfaces in Panel (*B*).

with several major differences. The elements of the paradigm include: (*a*) combined-action assessment is most appropriate for complex systems in which a complete correct description of the mechanisms by which the agents cause their single and joint effects does not exist. If such a description does exist, then mathematical models based upon a mechanistic understanding of the concentration-effect relationships should be applied to data, not general combined-action mathematical models; (*b*) the degree of departure from "no interaction" of the concentration-effect surface for an agent combination is a quantitative measure of the ignorance of the investigator, i.e., if the system were well understood by the investigator, and this understanding were incorporated into the "no interaction" model, then the experimental results would be as predicted (e.g., Loewe additivity)—no more, no less; (*c*) the Loewe additivity equation, Eq. 6, the Bliss independence equation, Eq. 11 or 14, or response surface interaction models adapted directly from them, should be used in an initial step to evaluate departures from the no interaction reference, without regard to mechanistic interpretation; (*d*) a later useful step in interaction assessment may involve the interpretation of Loewe synergism, Loewe additivity, Loewe antagonism, Bliss synergism, Bliss independence, Bliss antagonism, synergism, inertism, antagonism, or coalism via mechanistic arguments. For relatively simple systems, such as individual enzymes or receptors or small networks of enzymes and receptors, it may be useful to establish the relationship between empirical interaction models and mechanistic biochemical models. However, except for very well understood simple systems, it is unlikely that the results of a combined-action analysis will unambiguously lead to a correct mechanistic explanation of an observed agent interaction; (*e*) the main uses of general combined-action analyses are:

(1) to summarize a large amount of data with a joint concentration-effect surface, with relatively few parameters, for a combination of agents.

(2) to facilitate good predictions of joint effects in regions in which no real data was collected (interpolation and judicious extrapolation).

(3) to empirically find and characterize agent combinations with intense interactions, in order to use or to avoid the combinations for specific practical purposes.

(4) to quantitatively characterize a system, so that the effect of changes in some other factor can be quantified.

(5) to provide a lead to a mechanistic explanation of joint action.

IV. Debate Over the Best Reference Model for Combined-action

Because synergism (and antagonism) are commonly defined as a greater (or lesser) pharmacological effect for a two-drug combination than what would be predicted for "no interaction" from the knowledge of the effects of each drug individually, their definitions critically depend upon the reference model for "no interaction." It is our view that there are only two reference models that deserve extensive consideration. The first, and our preference, is Loewe additivity, which is defined by Eq. 6. A specific model for Loewe additivity that assumes the Hill equation, Eq. 2, for the concentration-effect model for each drug individually, is Eq. 13.

$$1 = \frac{D_1}{IC_{50,1}\left(\dfrac{E}{Econ - E}\right)^{1/m_1}} + \frac{D_2}{IC_{50,2}\left(\dfrac{E}{Econ - E}\right)^{1/m_2}} \qquad [13]$$

Note that Eq. 13 is equivalent to Eq. 5 with the third right-hand term, the interaction expression, dropped. Also note that Eq. 13 is merely the Loewe additivity model, Eq. 6, with the substitution of the definition of ID_X for the Hill model, Eq. 7, for both drugs. This derivation for a specific Loewe additivity model follows the guidelines of Berenbaum (1985), and the examples of Hewlett (1969) and Sühnel (1992c). The additivity reference concept was first mentioned by Frei (1913) and was first defined formally by Loewe and Muischnek (1926). The Loewe additivity reference is the diagonal Northwest-Southeast line in isobolograms of figures 4, 5, 6, and 8 and is a key part of the classical isobologram approach (Loewe and Muischnek, 1926; Elion et al., 1954; Gessner, 1974).

The simplest intuitive explanation of the concept of Loewe additivity is the following sham experiment: an aliquot of a solution of drug 1 from a tube is poured into a second tube and then diluted with an appropriate solvent. When these two preparations are falsely labeled as different agents and their combination is examined, the result will be Loewe additivity. [Gennings et al. (1990) experimentally illustrated and verified this concept by examining the loss of righting reflex of mice treated with the combination of sodium hexobarbital with itself.] Thus, by definition, one agent is noninteractive with itself. Advocates of the Loewe additivity reference for no interaction use this sham study of one drug with itself as a litmus test to invalidate other reference models (e.g., Berenbaum, 1981). From this logic, Loewe additivity implies that each of two drugs act similarly, presumably at the same site of action, differing only in potency. However, Eq. 6 is less restrictive than this narrow interpretation. The constraint of Eq. 6 can be obeyed for two drugs with different concentration-effect slopes, (e.g., Eq. 13) that presumably would not act at the same site. In fact, each of the two drugs in a combination could follow different concentration-effect functions and still obey Loewe additivity, Eq. 6. This flexibility is considered a weakness, with no theoretical justification, by opponents of the Loewe additivity reference [Greco et al., (1992)]. They contend that the rare observation of Loewe additivity in real complex experimental systems is only fortuitous and does not lead one to any mechanistic conclusion.

The strongest advocate of approaches based upon the Loewe additivity reference has been Berenbaum (1977, 1978, 1981, 1985, 1988, 1989). Of the approaches evaluated in our review, the following use the Loewe additivity reference: isobologram by hand; interaction index of Berenbaum (1977); median-effect method of Chou and Talalay (1984); mutually exclusive model method of Berenbaum (1985); bivariate spline fitting (Sühnel, 1990); parametric response surface approaches of Greco et al. (1990) and Weinstein et al. (1990); approach of Gessner (1974); parametric response surface approach of Greco and Lawrence (1988); and the use of the multivariate linear logistic model (Carter et al., 1983, 1986, 1988; Brunden et al., 1988). The concepts of similar joint action (Bliss, 1939), simple similar action (Plackett and Hewlett, 1952), and concentration (dose) addition (Shelton and Weber, 1981) are all consistent with Loewe additivity, as defined by Eq. 6. However, as discussed above, Loewe additivity also includes cases not consistent with these more restrictive concepts.

In our view, the most convincing argument in favor of the use of the Loewe additivity model, Eq. 6, as a universal reference to define "synergism" and "antagonism," is that it can best survive criticism. With the possible exception of Bliss independence, all of the other candidate reference models can be fatally wounded from well aimed attacks; whereas, the Loewe additivity model, although not completely unscathed, is still standing after the smoke of battle clears. The Loewe additivity reference model, by definition, yields the intuitive correct evaluation of the sham combination of one drug with itself to be Loewe additivity (or as preferred by Berenbaum, 1981, "no interaction"). The Loewe additivity reference model is, in fact, merely a reasonable assumption. The interpretation of an assessment of Loewe additivity, Loewe synergism, or Loewe antagonism is, in general, free of mechanistic restrictions and implications. [In principle, the mathematical models and parameters of specific biological systems can be mapped to empirical combined-action models and parameters to facilitate a mechanistic interpretation of a combined-action analysis, but work on such mappings is in its infancy (e.g., Bravo et al., 1992; Jackson, 1993).] From a response surface perspective, the Loewe additivity model, Eq. 6, can be adapted to yield many useful empirical models of combined-action, such as Eq. 5.

In our view, the only other major contender for a universal reference of noninteraction (worthy of the silver medal) is Bliss independence, Eq. 11, or its equivalents. Eq. 12 is a specific Bliss independence model that assumes that the Hill model, Eq. 2, is an appropriate concentration-effect model for each drug individually. Bliss independence implies that two agents do not physically or chemically or biologically cooperate; i.e., each agent acts independently of the other. Berenbaum (1981) describes an interesting hypothetical experiment that provides an intuitive feel for independently acting agents. His thought experiment involves randomly throwing either bushels of nails or pebbles or both at a collection of eggs. None of the causal units, nails or pebbles, cooperate with each other in the cracking of an egg, an all-or-none phenomenon. But rather, each causal unit has a certain probability (different for nails or pebbles) of hitting an egg, and the cumulative damage is merely the result of correctly combining probabilities.

The Bliss independence reference model has an intuitive, theoretical basis: the concept of noninteraction; it has a simple general formula, Eq. 11. Testing of the model usually requires frugal experimental designs, and many specific approaches for interaction assessment incorporate it. These approaches include: the fractional product method of Webb (1963); the method of Valeriote and Lin (1975); the method of Drewinko et al. (1976); the method of Steel and Peckham (1979), Mode I; and the method of Prichard and Shipman (1990). Synonyms for Bliss independence include: independent effects, independent joint action (Bliss, 1939); independent action (Plackett and Hewlett, 1952); response (effect) addition (Shelton and Weber, 1981); effect summation (Gessner, 1988); and effect multiplication (Berenbaum, 1981). [Note: if Eq. 11 is recast in terms of the fraction of possible effect, with subscripts referring to specific concentrations of agent 1, agent 2, and the corresponding combination of agents 1 and 2, then Eq. 14 is the result. This equation is analogous to the common formula for the combination of probabilities (e.g., Larson, 1982).]

$$fa_{12} = fa_1 + fa_2 - fa_1 fa_2 \qquad [14]$$

Gessner (1974; 1988) offered a philosophical argument against the Bliss independence model: *he questioned whether, given the high degree of integration of a living organism, the action of an agent on one receptor type, target organ, or system can ever be envisaged as not altering to some degree the responsiveness of other receptors, organs, or systems to a simultaneously present second agent.* Certainly, complex systems with extensive positive and negative feedback pathways at all levels of biological organization are ubiquitous and are the chief targets of drug therapy (Jackson, 1992). Most examples of theoretical systems that follow the Bliss independence model are relatively simple, such as single enzymes (e.g., Webb, 1963) and simple biochemical pathways (e.g., Jackson, 1991).

Gessner (1988) also mentioned that he had never seen a published isobologram for the 50% effect level for quantal data in which an isobol reasonably followed the Bliss independence model throughout the whole curve. In contrast, Pöch and coworkers have reported several examples of Bliss independence (e.g., Pöch,1990; Pöch et al., 1990a, b, c; Pöch, 1991; Pöch, 1993). An objective survey would be necessary to estimate the frequency of occurrence of exact Bliss independence for combinations of agents in real experimental work. However, just as with Loewe additivity, it is also our impression that pure Bliss independence in complex systems is a rare occurrence.

The most convincing arguments against the Bliss independence model as a universal reference model for noninteraction use the pair of concentration-effect curves in figure 11 (Greco, 1989). [Similar figures and arguments were previously published by Grindey et al. (1975) and Berenbaum (1977, 1981)]. Figure 11 includes individual simulated data points and simulated concentration-effect curves for two different hypothetical inhibitory drugs. Suppose that 0.5 μM of drug 1 results in 95%

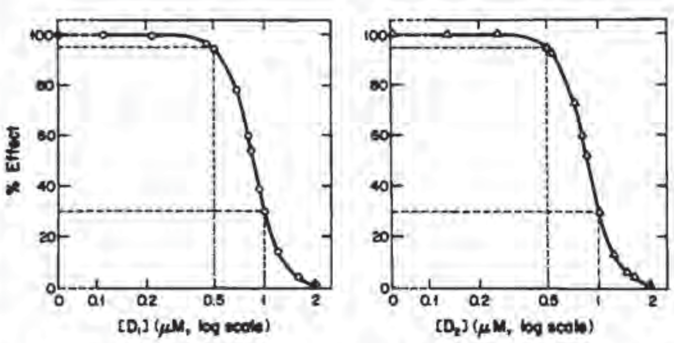


FIG. 11. Hypothetical concentration-effect curves for two drugs to demonstrate a logical inconsistency for approaches to assess drug synergism based upon the assumption of Bliss independence, Eq. 11 or Eq. 14, as the "no interaction" reference model.

survival of cells in a typical growth inhibition experiment, likewise for drug 2. From Eq. 11, one would predict that the noninteractive response for 0.5 μM of drug 1 plus 0.5 μM of drug 2 would be about 90% survival. Therefore, if one found that this drug combination elicited, let's say, 40% survival of cells, one would conclude strong, undeniable Bliss synergism. However, note in figure 11 that either 1 μM of drug 1 alone or 1 μM of drug 2 alone brings the survival of cells down to 30%. Therefore, a total of 1 μM of the hypothetical combined drug preparation elicits less of a cell kill than 1 μM of either drug alone, yet one would conclude strong Bliss synergism under methods based upon the Bliss independence reference assumption, Eq. 11.

Figure 11 can also be used to illustrate the paradox of the sham combination of one drug with itself. Let's say that a drug preparation is divided into two tubes, and then each tube is treated as if it contained a different drug. The two concentration-effect curves in figure 11, which are in fact identical, would result. Using the same logic as used in the beginning of the previous paragraph, one would conclude that the drug is Bliss synergistic with itself. This absurd conclusion is inconsistent with the intuitive definitions of "synergism," "additivity," and "antagonism" used by many researchers.

It is our view that these two aspects of the same basic criticism illustrated by figure 11 are persuasive enough to relegate Bliss independence to second place for the optimal routine reference for defining "synergism" and "antagonism." However, proponents of the Bliss independence reference have several counterarguments: (*a*) when concentration-effect curves are steep, such as in figure 11, the joint effects of a Bliss synergistic combination may be disappointingly small relative to the effects of each drug individually, but this result is neither paradoxical nor absurd; (*b*) a drug with a steep concentration-effect curve is Bliss synergistic with itself (this is a fundamental tenet of Biology); (*c*) the sham combination of a drug with itself is a silly experiment, and the so-called paradox is, at worst, a minor exception to a generally useful concept; (*d*) if it is known that two drugs in a combination act at the same biochemical site, a relatively rare situation, then their actions cannot be independent, and one shouldn't use the Bliss independence reference. Figure 11 is merely an illustration of the extreme case of this situation, in which the two dose response curves are identical.

Our rejoinders to these counterarguments include: (*a*) the search for synergy will often involve agents, drugs, and preparations with multiple, complex, possibly unknown mechanisms of action, and therefore, guidelines for the assessment of interaction must not depend upon knowledge of mechanisms of action; (*b*) a general concept must encompass rare cases; (*c*) the first argument illustrated by figure 11 did not require that the two drugs be the same or that they have similar sites of action, but only that they have steep concentration-effect curves; (*d*) a reference model that can result in the counterintuitive result, that a synergistic combination is less effective than its components applied individually, is not useful.

As pointed out by Berenbaum (1981), the fundamental explanation underlying both forms of the above paradox involves the functional form of the individual concentration-effect curves. Only when each individual concentration-effect curve follows Eq. 4, that for exponential decline with dose, will there be no paradox: Loewe additivity will be concluded from the sham combination of one drug with itself. Eq. 15 would be the resulting equation for no interaction of two drugs, from combining either Eq. 4 and Eq. 6 (Loewe additivity) or Eq. 4 and Eq. 11 (Bliss independence). Concentration-effect curves steeper than the exponential model will lead to the above paradox; whereas, concentration-effect curves less steep than the exponential model will lead to an opposite paradox. (Note: the data points and curves in figure 11 were simulated with Eq. 2 with $Econ$ = 100, IC_{50} = 0.86 μM, and m = -5.6, resulting in relatively steep curves.)

$$E = Econ \exp(aD_1)\exp(bD_2) = Econ \exp(aD_1 + bD_2) \qquad [15]$$

However, we disagree with Berenbaum's inference from the above logic that the Bliss independence model, Eq. 11, is appropriate for describing the joint action of a combination only when each of the component drugs have exponential concentration-effect curves, Eq. 4. Berenbaum (1981) argues that in order for molecules of drug 1 to act independently from molecules of drug 2, all molecules of drug 1 must act independently of all other molecules of drug 1, resulting in an exponential concentration-effect curve for drug 1; all molecules of drug 2 must act independently of all other molecules of drug 2, resulting in an exponential concentration-effect curve for drug 2. This argument can be refuted by a specific counterexample from Jackson (1991). Jackson (1991) modeled a hypothetical biochemical pathway consisting of: a substrate, A, being converted to substrate B by enzyme 1; substrate B being converted to substrate C by enzyme 2, and to substrate D by enzyme 3; a competitive inhibitor of enzyme 1; and a competitive inhibitor of enzyme 2. When the enzyme kinetic parameters are adjusted to give a high sink capacity (the ratio of the sum of the maximal velocities of enzymes 2 and 3 divided by the maximal velocity of enzyme 1), exact Bliss independence of the effects of the two inhibitors can be achieved. The individual concentration-effect curves for the two inhibitors followed the Hill model, Eq. 2, and thus were nonexponential, yet the specific Bliss independence model, Eq. 12, fit the data perfectly over a wide range of inhibitor concentrations (Bravo et al., 1992). In addition, Pöch (1991) provides several specific examples of Bliss independence found with real laboratory data, in which the individual concentration-effect curves follow the Hill model, Eq. 2 or 3. Thus, this specific argument of Berenbaum against the independent effects model is questionable.

Although we prefer Loewe additivity to Bliss independence as a universal reference for the lack of "synergism" or "antagonism," we must concede that the Bliss independence camp has successfully resisted total defeat. It is clear that adherents of Loewe additivity and Bliss independence have heard all of the most compelling arguments for and against each model and cannot be persuaded to switch allegiances. Thus, the debate can progress no further, and we join in the recommendation that both models be accepted as legitimate empirical reference standards for "no interaction." It must be emphasized, however, that neither model is well suited for unambiguously indicating mechanistic explanations for the joint action of agents in complex systems, such as whole cells, single organisms, or populations of organisms. In order for researchers to make mechanistic conclusions for a specific experimental system, the correspondence between empirical concepts—such as Loewe synergism or Bliss antagonism—and theoretical mechanisms must be derived. This is a rich source for future research initiatives.

The shapes of isobols for Loewe additivity and Bliss independence will, in general, be very different. Figure 12 shows a set of isobols at the 50% effect level for the specific Bliss independence model, Eq. 12, which incorporates the Hill model, Eq. 2, for the individual concentration-effect curves. The shape of the isobols is determined only by the two slope parameters, m_1 and m_2; these are listed in figure 12 next to each respective isobol. [Note: Similar figures and observations are provided by Gessner (1988) and Pöch et al. (1990c)]. When the slope parameters are the same for the two drugs, the isobols are symmetrical; when they are different, the isobols have an S shape and may cross the Loewe additivity diagonal. Slope parameters that are large in magnitude result in Loewe antagonism; whereas, slope parameters that are small in magnitude result in Loewe synergism. It may be useful to superimpose the predicted Bliss independence model on both 2-D and 3-D representations of two-drug combination concentration-effect surfaces. If the superimposed Bliss independence curves lie close to the data, then it may be useful to infer, after making necessary assumptions, that the two drugs may, in some sense, act independently.

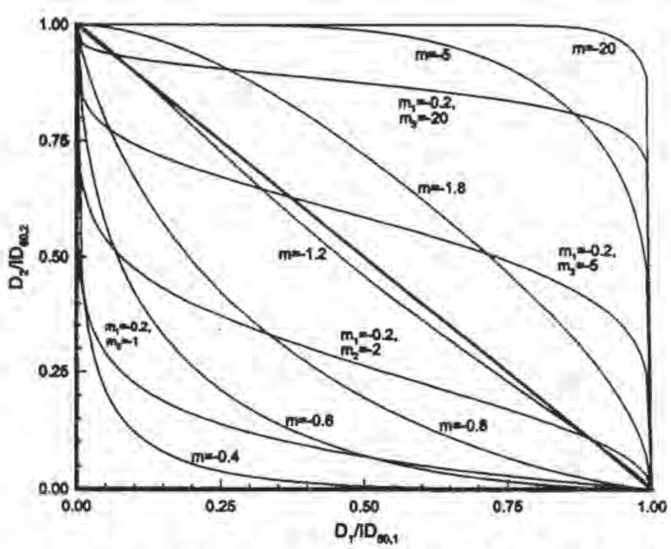


FIG. 12. Normalized isobols at the 50% effect level, for the Bliss independence model, Eq. 12, for various values of m_1 and m_2, which are set next to each corresponding curve. A single m indicates that $m_1 = m_2 = m$. The thick diagonal line is the line of Loewe additivity.

Four other candidates for a universal reference for no interaction will be briefly described and critiqued below. The first is Eq. 16, that for effect addition, and the second is almost the same, Eq. 17, that for fractional effect addition. [Note: Some authors call Eq. 11 and 14 the effect addition model (e.g., Shelton and Weber, 1981).]

$$E_{12} = E_1 + E_2 \qquad [16]$$

$$fa_{12} = fa_1 + fa_2 \qquad [17]$$

According to Eq. 16, if the effect for a particular concentration of drug 1 was 20 units and that for a particular dose of drug 2 was 30 units, then the no interaction prediction would be 50 units. As pointed out by Berenbaum (1981), this intuitive definition of no interaction may underlie the claims of synergism and antagonism for which authors provide no explicit definitions. Eq. 16 is not easily applied to the common case in which the drugs have some maximum possible effect, because if E_1 and E_2 are both reasonably large, 60 and 70, let's say, and close to the maximum possible effect, 100, let's say, then E_{12} would be 130, greater than the maximum possible effect, resulting in an inconsistency. For one restricted situation, when each of the individual concentration-effect curves are linear and increasing, Berenbaum (1981) showed that Eq. 16 is consistent with the Loewe additivity model, Eq. 6.

A somewhat more credible variation of effect addition, Eq. 16, is fractional effect addition, Eq. 17. According to Eq. 17, if the fraction of possible effect affected for drug 1 is 0.20 and the fraction affected for drug 2 is 0.30, then the no interaction prediction would be 0.50. Eq. 17 is also easily eliminated as a candidate for a universal standard by considering an example in which the fractional effects are both large, let's say, $fa_1 = 0.60$ and $fa_2 = 0.70$. Because fa_{12} has an upper limit of 1.0, the sum of $fa_1 + fa_2$, which equals 1.30, leads to an inconsistency. In addition, paradoxes regarding synergy, similar to those described above for the Bliss independence reference model, can be contrived using figure 11. However, Eq. 17 is valid or approximately valid under several restricted situations. The first is the case in which fa_1 and fa_2 are both very small. Then Eq. 17 will approximate Eq. 14, that for Bliss independence, because the product term will be very small (Pöch, 1991). The second is independent effects for quantal responses, in which the susceptibilities of the individual organisms to the two drugs are completely negatively correlated (any organism that is affected by drug 1 will not be affected by drug 2, and vice versa) (Plackett and Hewlett, 1948). The third is the joint effects of two inhibitors in a metabolic network in which two converging reactions that lead to a single product are both inhibited (Jackson, 1991). Note that these latter two examples of restricted conditions both impose upper limits upon the magnitudes of fa_1 and fa_2; their sum never exceeds 1.0.

Another candidate for a universal reference for no interaction is the mutually nonexclusive model of Chou and Talalay (1984), Eq. 18. An alternate form is Eq. 19, which is equivalent to Eq. 5, our model for drug interaction, with $m = m_1 = m_2$ and $\alpha = 1$. As emphasized by Chou and Talalay (1984), their mutually nonexclusive model is equivalent to the Bliss independence model only under restricted conditions; specifically, when the median-effect model (equivalent to Eq. 1 or Eq. 2) adequately describes the individual concentration-effect curves for both drugs and $m_1 = m_2 = -1$ for monotonically decreasing curves [or $m_1 = m_2 = 1$ for monotonically increasing curves, as preferred by Chou and Talalay (1984)]. They further conclude that the Bliss independence model is inadequate under conditions in which $|m| \neq 1$. However, it is our view that it is the mutually nonexclusive model that is suspect. Only an abbreviated general derivation of this model, for the case of multiple mutually nonexclusive inhibitors of a single enzyme, is provided in Chou and Talalay (1981). A specific derivation, for the case of two mutually nonexclusive noncompetitive inhibitors, is provided in Appendix A. An equation equivalent to Eq. 12, not to Chou and Talalay's mutually nonexclusive model, is the result. Because their model is of questionable validity, we feel that it is not appropriate as a universal reference. An extensive discussion of the median-effect approach to the assessment of drug interaction is provided in Section V.G.

$$\left(\frac{fa_{12}}{fu_{12}}\right)^{1/m} = \left(\frac{fa_1}{fu_1}\right)^{1/m} + \left(\frac{fa_2}{fu_2}\right)^{1/m} + \left(\frac{fa_1 fa_2}{fu_1 fu_2}\right)^{1/m} \qquad [18]$$

$$= \frac{D_1}{Dm_1} + \frac{D_2}{Dm_2} + \frac{D_1 D_2}{Dm_1 Dm_2}$$

$$E = \frac{Econ\left(\frac{D_1}{IC_{50,1}} + \frac{D_2}{IC_{50,2}} + \frac{D_1 D_2}{IC_{50,1} IC_{50,2}}\right)^m}{1 + \left(\frac{D_1}{IC_{50,1}} + \frac{D_2}{IC_{50,2}} + \frac{D_1 D_2}{IC_{50,1} IC_{50,2}}\right)^m} \qquad [19]$$

A final candidate for a universal reference for no interaction is the Mode II additivity model of Steel and Peckham (1979). A compact way to express the model is Eq. 20. An equivalent form is provided by Kodell and Pounds (1991).

$$D_2 = ID_{[X - fa(D_1)],2} \qquad [20]$$

Eq. 20 can be used to construct an isobol for D_2 versus D_1 for a particular $X\%$ inhibition. To do this, D_1 is varied, and the fraction affected (% inhibition) for the particular D_1 is calculated and subtracted from the target $X\%$. Then, the D_2 needed to achieve this resulting difference $X\%$ is determined. Interestingly, this reference model will give the correct answer of no interaction for a sham combination of drug 1 with itself; the isobol will be a straight diagonal NW-SE line, such as in figure 6. However, Eq. 20 is not equivalent to the Loewe additivity model, Eq. 6. This will be shown and discussed in detail in Section V.F. A fatal flaw of the Mode II reference model is that it has a polarity; i.e., for two different drugs, different isobols will be drawn, depending upon the arbitrary assignment of drug 1 and drug 2 (Berenbaum, 1981).

The issue of the preferred reference model for no interaction has been recently debated in the antiviral literature by Sühnel (1990; 1992a) and Prichard and Shipman (1990; 1992). We endorse Sühnel's advocacy of the Loewe additivity model, Eq. 6 over Prichard and Shipman's advocacy of Bliss independence, Eq. 11 or Eq. 14. However, this is mainly because of personal preference and because our specific response surface models incorporate Loewe additivity. We do not endorse Sühnel (1990, 1992a) and Berenbaum's (1981) main argument that the Bliss independence model is only valid for the case in which each individual concentration-effect curve follows an exponential concentration-effect curve. Rather, we feel that the paradoxes illustrated with figure 11 are sufficient to place Bliss independence in second place for the competition for a universal null reference model.

In summary, we advocate the use of the Loewe additivity model, Eq. 6, as the best choice for a universal standard reference for defining "synergism" and "antagonism." Adaptations of Eq. 6 can be used to derive concentration-effect response surface functions, such as Eq. 5, containing interaction parameters, such as α. To the best of our knowledge, response surface models for agent interaction that incorporate Bliss independence have not been developed. However, some ideas of Ashford and Smith (1964) and Ashford (1981), which have been recently reviewed by Unkelbach (1992), have the potential to lead to the development of such models.

V. Comparison of Rival Approaches for Continuous Response Data

There are many published methods for assessing drug interactions. We have carefully chosen 13 of them for continuous response data to compare in a head-to-head competition. (Section VI includes a comparison of three rival approaches for discrete success/failure data.) Some methods consist of general guidelines, whereas others include very specific recipes. This set of 13 methods was chosen because, as a group, they have a high frequency of use, have a high relative impact on biomedicine, have many similarities and differences, provide a good summary of the practical history of drug interactions, include good examples of the pleasures, pitfalls, controversies and paradoxes inherent in the field, and point toward the future of interaction assessment. Noteworthy additional approaches not extensively evaluated in this review include the ones by Pöch (1990b), Kodell and Pounds (1985), Tallarida et al. (1989), Kelly and Rice (1990), and Laska et al. (1994). The 13 rival approaches will be compared in two ways: (*a*) Theoretical aspects, both positive and negative, of each approach will be listed and discussed. Although a large number of these comments will be summarized from previous work of other reviews, there will be many new comments. Several of the theoretical comments will refer back to Sections I to IV. (*b*) An abbreviated recipe for the application of each approach to a common data set, for an inhibitory drug, will be described. For a complete recipe of each approach, the reader is encouraged to consult the original references. Each approach will then be applied to a common data set. Pitfalls, problems, and results will be listed and compared.

The common data set consists of the 38 data points in columns 2 to 4 of table 3, simulated with the approach described completely in footnote *a* of the table. Briefly, this data set was simulated with Eq. 5 as the structural model, with different slope parameters for the two drugs (m_1 = -1, m_2 = -2) and with a small amount of synergism (α = 0.5). The data contains normally distributed random relative errors; the coefficient of variation is 10%. A simulated Monte Carlo data set was used, as opposed to a real data set, because: (*a*) the "true" answer is known, so there is an absolute reference for making comparisons between rival approaches; and (*b*) specific characteristics can be imbedded in the data set to illustrate specific differences among rival approaches. To the best of our knowledge, this approach to making comparisons among rival methods to assess agent interaction has not been used by groups other than ours (Syracuse and Greco, 1986; Greco, 1989).

A. Isobologram by Hand

The graphical isobologram approach, performed by hand, with the aid of pencil, ruler, graph paper, and possibly French curve, has its origins in the work of Fraser (1870–1871; 1872), Loewe (Loewe and Muischnek, 1926; Loewe, 1928, 1953, 1957), and Elion, Singer, and Hitchings (1954). It is a general approach and has many interpretations and variants. Our interpretation is described here. The first step is to plot the measured data, such as those found in columns 2 to 4 of table 3, as concentration-effect curves, such as in figure 13. Two separate graphs are drawn, usually by hand with a French curve or a straight edge, one for drug 1 and the other for drug 2. Each graph has a family of concentration-effect curves, one curve for each level of the other drug. The IC_{50} (or Dm, ID_{50}, ED_{50}, LD_{50}, etc.) values are then determined, by eye, for each curve on both graphs. From Figure 13, six IC_{50} values can be determined, three from the left panel and three from the right panel. (An IC_{50} value cannot be determined for six of the concentration-effect curves, because for each of them, the measured response at the first drug concentration is already below 50% of the maximum measured response.) From the left panel, the IC_{50} values for drug 1 are recorded along with the level of drug 2 used to generate the respective concentration-effect curves. Then, these IC_{50} values for drug 1 are divided by the IC_{50} value for drug 1 in the absence of drug 2, and the levels of drug 2 are divided by the IC_{50} for drug 2 alone. The resulting data points, (D_1/Dm_1, D_2/Dm_2), are the solid points on the left isobologram of figure 14. The analogous procedure is performed on the concentration-effect curves of the right panel of figure 13, resulting in the open points in the left panel of figure 14. In the isobolograms of figure 14, each data point is labeled (a-l) to correspond to the curve in figure 13 from which it was derived. Occasionally, smooth curves are drawn through points on an isobologram, possibly with a French curve; occasionally, straight lines are drawn connecting the points, and occasionally, no curve is drawn at all. In figure 14, curve W is not a curve drawn by hand, but rather is the theoretically correct isobol simulated with Eq. 21 (an isobol model that assumes that Eq. 5 is appropriate for the entire concentration-effect surface), for the 50% level and for α = 0.5. As explained in Section III and shown in figures 4, 5, 6, and 8, the diagonal NW-SE line is the line of Loewe additivity; points below the line indicate Loewe synergism and points above the line indicate Loewe antagonism.

$$\frac{D_2}{IC_{X,2}} = \frac{1 - \dfrac{D_1}{IC_{X,1}}}{1 + \dfrac{\alpha D_1}{IC_{X,1}}\left[\dfrac{100 - X}{X}\right]^{(1/2m_1 + 1/2m_2)}} \qquad [21]$$

In principle, any constant effect level can be used for an isobologram analysis, not just the 50% level. Because most of the concentration-effect curves from figure 13 did not yield a Dm value, IC_{80} (D_{80}) values were also determined. The right panel of figure 14 is the isobologram analysis of the D_{80} values.

If one only used the Dm isobologram from figure 14, one would conclude that the experiment should be repeated. If one also used the D_{80} isobologram from figure 14, one would conclude that the interaction between drug 1 and drug 2 is Loewe synergistic.

TABLE 3

Data set, with a continuous response variable, used for comparison of rival data analysis approaches, and the results from four approaches

Data point number	D_1	D_2	Measured effect*	Predicted effect from Bliss independence model†	Conclusion from fractional product comparison‡	Conclusion from V and L system§	Drewinko's Score‖	Predicted effect from Loewe additivity model¶	Berenbaum's interaction index, I#	Conclusion from Loewe additivity comparison**
1	0	0	106					99.2		
2	0	0	99.2					99.2		
3	0	0	115					99.2		
4	0	0.2	79.2					94.6		
5	0	0.5	70.1					77.4		
6	0	1	49.0					47.9		
7	0	2	21.0					19.6		
8	0	5	3.83					4.00		
9	2	0	74.2					81.7		
10	5	0	71.5					64.9		
11	10	0	48.1					48.4		
12	20	0	30.9					32.2		
13	50	0	16.3					16.1		
14	2	0.2	76.3	55.0	BANT	INT	21.3	74.3	1.10	LANT
15	2	0.5	48.8	48.6	BANT	SUB	0.2	61.1	0.713	LSYN
16	2	1	44.5	34.0	BANT	SUB	10.5	40.6	1.10	LANT
17	2	2	15.5	14.6	BANT	SUB	0.9	18.2	0.901	LSYN
18	2	5	3.21	2.66	BANT	SUB	0.55	3.94	0.895	LSYN
19	5	0.2	56.7	52.9	BANT	SUB	3.8	58.3	0.944	LSYN
20	5	0.5	47.5	46.9	BANT	SUB	0.6	48.2	0.978	LSYN
21	5	1	26.8	32.7	BSYN	BSYN	−5.9	33.6	0.811	LSYN
22	5	2	16.9	14.0	BANT	SUB	2.9	16.5	1.02	LANT
23	5	5	3.25	2.56	BANT	SUB	0.69	3.85	0.911	LSYN
24	10	0.2	46.7	35.6	BANT	SUB	11.1	43.5	1.13	LANT
25	10	0.5	35.6	31.5	BANT	SUB	4.1	36.5	0.968	LSYN
26	10	1	21.5	22.1	BSYN	BSYN	−0.6	26.7	0.818	LSYN
27	10	2	11.1	9.44	BANT	SUB	1.66	14.4	0.836	LSYN
28	10	5	2.94	1.72	BANT	SUB	1.22	3.72	0.878	LSYN
29	20	0.2	24.8	22.9	BANT	SUB	1.9	29.2	0.809	LSYN
30	20	0.5	21.6	20.3	BANT	SUB	1.3	25.1	0.844	LSYN
31	20	1	17.3	14.1	BANT	SUB	3.2	19.4	0.899	LSYN
32	20	2	7.78	6.07	BANT	SUB	1.71	11.6	0.751	LSYN
33	20	5	1.84	1.10	BANT	SUB	0.74	3.47	0.698	LSYN
34	50	0.2	13.6	11.3	BANT	SUB	2.3	15.0	0.898	LSYN
35	50	0.5	11.1	9.96	BANT	SUB	1.14	13.4	0.824	LSYN
36	50	1	6.43	7.47	BSYN	BSYN	−1.04	11.1	0.613	LSYN
37	50	2	3.34	3.20	BANT	SUB	0.14	7.66	0.539	LSYN
38	50	5	0.890	0.583	BANT	SUB	0.307	2.93	0.496	LSYN
				Totals	BSYN = 3 BANT = 22	BSYN = 3 SUB = 21 INT = 1	mean = 2.59 S.D. = 5.1 S.E. = 1.02			LSYN = 21 LANT = 4

* The "Measured Effects" were generated by: (*a*) calculating ideal data with Eq. 5 with parameters, $Econ = 100$, $IC_{50,1} = 10$, $IC_{50,2} = 1$, $m_1 = -1$, $m_2 = -2$, $\alpha = 0.5$; (*b*) generating normally distributed random numbers with a mean of 0 and a variance of 1 (Box and Müller, 1958); (*c*) calculating relative errors by the equation, error = [(normal random number)/10] × [ideal effect]; (*d*) adding the errors to the ideal effects to generate simulated data with relative error (a coefficient of variation of 10%).

† Each measured effect from column 4 was divided by the average of the control effects (107) to yield a fraction of control effect, then the fractional effects for the appropriate D_1 and D_2 were multiplied, then this product was multiplied by the average of control effects to yield the entries in column 5.

‡ For the fractional product approach to the assessment of drug interaction (Webb, 1963), when the entry in column 4, the measured effect, is greater than the entry in column 5, the predicted effect, then Bliss antagonism (BANT) (less inhibition than predicted) is recorded; when the column 4 entry is less than the column 5 entry (more inhibition than predicted), then Bliss synergism (BSYN) is recorded.

§ The Valeriote and Lin (1975) system differs from the Webb approach by further subdividing the Bliss antagonism into 3 categories, subadditivity (SUB), interference (INT), and antagonism (ANT). Details are in the text.

‖ Column 8 is the difference between columns 4 and 5. From the mean and standard error of the mean for this difference score (Drewinko et al., 1976) one would conclude significant antagonism ($P < 0.05$). See the text for details.

¶ The predictions in column 9 are based on the best fit of Eq. 13 to the data points for which drug 1 and drug 2 were not simultaneously present, i.e., the data in columns 2–4, rows 1–13, and then the simulation of Eq. 13 with these 5 best fit parameters for all of the 38 data points.

\# Berenbaum's interaction index (I) is calculated from Eq. 22, with the ID_Xs for drug 1 and drug 2 calculated from Eq. 7 with the parameter values from the best fit of Eq. 13 to the first 13 data points.

** When $I > 1$, then Loewe antagonism (LANT) is concluded; when $I < 1$, then Loewe synergism (LSYN) is concluded.

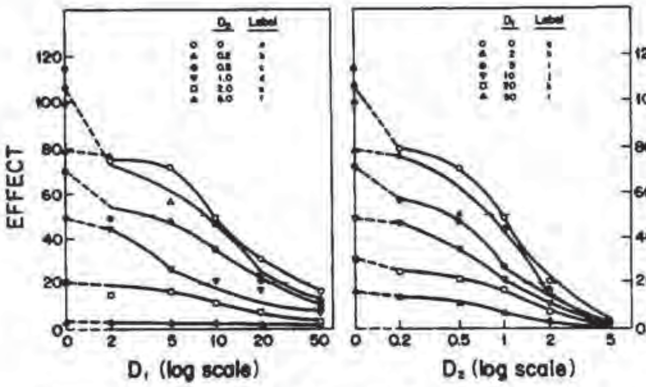


FIG. 13. Hand-drawn (with the aid of a French curve) concentration-effect curves for the data in columns 2 through 4 from table 3. The IC_{50} and IC_{80} values for each curve are indicated by short horizontal lines intersecting the curves.

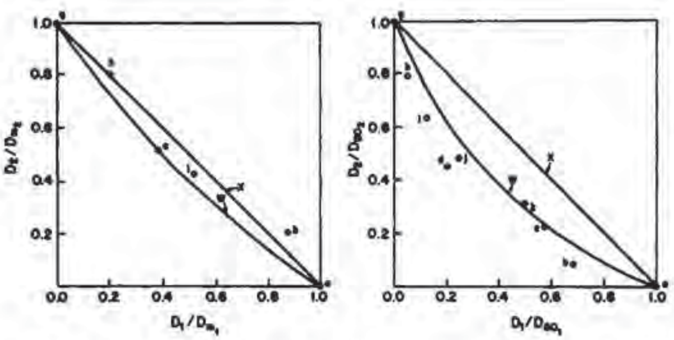


FIG. 14. Isobolograms made from IC_{50} values (left panel) and IC_{80} values (right panel). Line x in each panel is the Loewe additivity line. The data points in each panel are labeled with a lowercase letter that corresponds to the appropriate curve from figure 13. The solid points were derived from the left panel of figure 13, and the open points from the right panel. Curves W in each panel of figure 14 are the theoretically correct isobols and were simulated from Eq. 21 with parameters: $Econ = 100, IC_{50,1} = 10, IC_{50,2} = 1, m_1 = -1, m_2 = -2, \alpha = 0.5$.

The advantages of the isobologram by hand method include:

(*a*) the null reference model for no interaction is the Loewe additivity model, Eq. 6, which was given support in Section IV and is our preferred universal standard.

(*b*) the approach is simple, flexible, and to many users, intuitive.

(*c*) equipment to run the approach is inexpensive, and expert statistical advice and/or the learning of some modern statistical ideas are unnecessary.

(*d*) the approach is famous and widely accepted.

(*e*) variants of the basic method exist that add more statistical rigor (e.g., Gessner, 1974; Gennings et al., 1990) and that provide quantitative measures of interaction intensity (e.g., Hewlett, 1969; Elion et al., 1954; Pöch, 1980).

(*f*) many newer, more rigorous methods have the basic isobologram approach as their underlying basis (e.g., the method of Berenbaum (1985), the nonparametric bivariate spline fitting approach of Sühnel (1990), and the parametric response surface approach of Greco et al. (1990).

The disadvantages of the isobologram by hand method include:

(*a*) the method lacks many of the good characteristics of objective statistical procedures. It lacks the theoretical framework to allow inferences with a specified degree of certainty to be made from an experiment to the true situation. It lacks the option of objectively weighting more precise measurements greater than less precise ones.

(*b*) the basic isobologram method lacks a summary measure of the intensity of interaction.

(*c*) for the isobologram method, each concentration-effect curve should have data that encompasses the IC_X level. When this is not the case, such as with curves d-f, j-l in figure 13, for the 50% effect level, the data for those curves is wasted. If enough data is wasted, then the experiment may have to be rerun.

(*d*) in general, the basic isobologram method requires a relatively large amount of data. When data is expensive, combination experiments may become prohibitive.

(*e*) graphs of a measured dependent variable vs. an experimentally fixed independent variable, often fruitfully assumed to be recorded without error, are appealing, because they represent directly the actual experiment. Fitted curves can be superimposed upon actual observed data points to provide a good indication of the goodness of fit of the data by the curves. Isobolograms are not such graphs; no observed data points appear on them. Both the *X*- and *Y*-variables in isobolograms are subject to error of a complex, unknown distribution.

(*f*) the scatter of points in an isobologram may lead the researcher to a false conclusion of Loewe synergism in some regions and Loewe antagonism in other regions of the concentration-effect surface. Such a conclusion might be reached with the isobologram in the left panel of figure 14.

(*g*) it may take a relatively long time to plot by hand the required curves and to perform the required calculations.

(*h*) different data analysts are likely to plot the data differently and thus arrive at different answers.

B. Fractional Product Method of Webb (1963)

This method is a very simple one. Eq. 11, that for Bliss independence, is used to construct a set of predicted fractional responses, fu_{12}, as the product of the individual fractional effects, fu_1 and fu_2, for specific concentration combinations. Then, optionally, the results can be re-expressed as responses on the original response scale by multiplying each fu_{12} by the control response, as was done to calculate the entries for column 5 of table 3 for the analysis of the 38-point common data set. For an inhibitory drug, when the predicted response exceeds the measured response, Bliss synergism is claimed; when the measured response exceeds the predicted response, Bliss antagonism is claimed. Column 6 of table 3 lists the conclusions for each of the 25 combination points. There were 22 claims of Bliss antagonism and 3 claims of Bliss synergism. The overall conclusion is moderate Bliss antagonism, seemingly different from the conclusion of Loewe synergism from the isobologram analysis.

The advantages of the fractional product method include:

(*a*) it is the simplest of all methods; it is very intuitive. Calculations can be performed with pencil and paper; thus, equipment and personnel to run the method are inexpensive. The approach is famous and widely accepted.

(*b*) experimental designs can be very frugal; in principle, one can perform the experiment at single drug 1 and drug 2 concentrations, and thus one minimally needs only four data points to apply the method: (0, 0); (D_1, 0); (0, D_2); and (D_1, D_2).

(*c*) variants of the fractional product method exist that add some statistical rigor; e.g., the method of Steel and Peckham (1979) and the method of Prichard and Shipman (1990).

The disadvantages include:

(*a*) the no interaction null reference model for the fractional product method is the Bliss independence model, Eq. 11, which in our view, is slightly inferior to the Loewe additivity model, Eq. 6.

(*b*) the fractional product method is inconsistent with the isobologram method. It is possible to arrive at the opposite conclusion from that found with the isobologram method, as illustrated by the respective analyses of our common data set.

(*c*) there is no objective quantitative summary measure of the intensity of synergism or antagonism. It is not obvious how to combine results from several sets of measurements.

(*d*) a frugal design may give a misleading result if the pattern of interaction is different at different regions of the concentration-effect surface.

C. Method of Valeriote and Lin (1975)

This method is very similar to the fractional product method of Webb (1963). A predicted response is calculated from the Bliss independence null reference model; e.g., column 5 in table 3. Then, just as with Webb's method, the observed and predicted responses are compared. However, Valeriote and Lin (1975) further subdivide the less-than-additive region into subadditive, interference, and antagonism subregions. For an inhibitory drug, an interaction for a combination point is called (*a*) subadditive, if the surviving fraction is between predicted additivity and the surviving fraction for the more active drug, (*b*) interference, if the surviving fraction for the combination is between the observed surviving fractions of the two individual drugs, and (*c*) antagonism, if the surviving fraction for the combination is more than for the least potent drug.

The results from the application of the Valeriote and Lin (1975) approach to the common data set are: 3 combination points showed Bliss synergism, 21 points showed subadditivity, and 1 point showed interference. The conclusion is subadditivity.

The advantages and disadvantages of the Valeriote and Lin (1975) method are essentially the same as of the fractional product method of Webb (1963). The extra subdivision of the less-than-additive region into three regions may have merit.

D. Method of Drewinko et al. (1976)

This approach is also similar to the fractional product method of Webb (1963). The predicted surviving fraction is calculated from the Bliss independence model and listed as in column 5 of table 3. Then, the predicted surviving fraction is subtracted from the measured surviving fraction for the combination points, and the difference scores are listed, such as in column 8 of table 3. The scores are then used as data for a Student's t-test for the hypothesis that the true mean is equal to zero. For the 25 combination points for the common data set, the mean Drewinko score was 2.59, with a standard error of 1.02. There was significant Bliss antagonism, $P < 0.05$.

The advantages and disadvantages of the method of Drewinko et al. (1976) are essentially the same as those of the last two approaches. A difference is that this method offers a summary measure of the intensity of interaction, with an associated statistical indication of the uncertainty in the measure. A disadvantage of the mean Drewinko score is that it is not the statistical expectation of any specific true parameter. In other words, the mean Drewinko score will very much depend upon which regions of the concentration-effect surface are sampled. A statistic, such as the mean Drewinko score, that depends heavily upon the design of the experiment is not ideal.

E. Interaction Index Calculation of Berenbaum (1977)

This method is the algebraic analog of the isobologram by hand method. The general formula for the interaction index, I, is Eq. 22, in which D_1 and D_2 are concentrations of drug 1 and drug 2 in the combination, and $ID_{X,1}$, $ID_{X,2}$, are the predicted inhibitory concentrations of each drug individually to give the observed effect of the combination. The specific method of estimating $ID_{X,1}$ and $ID_{X,2}$ is left to the researcher but is often done by hand with pencil, graph paper, and possibly, French curve.

$$I = \frac{D_1}{IC_{X,1}} + \frac{D_2}{ID_{X,2}} \qquad [22]$$

We applied the interaction index method to the common data set by first fitting the first 13 data points with Eq. 13, that for Loewe additivity for two inhibitory drugs that both individually follow Eq. 2. The first 13 data points include the control points plus the drug 1 alone and drug 2 alone points. The data were fit with nonlinear regression, weighted by the reciprocal of the square of the predicted effect. (This weighting factor is appropriate for continuous data that have errors that are normally distributed and proportional to the true response. This error structure is common in biological systems and was used to generate the common data set, as described in the legend of table 3.) The 5 parameter estimates were: $Econ = 99.2 \pm 5.2$; $IC_{50,1} = 9.52 \pm 1.7$; $IC_{50,2} = 0.966 \pm 0.094$; $m_1 = -0.989 \pm 0.11$; $m_2 = -1.93 \pm 0.13$. Then, using Eq. 8, the specific form of Eq. 22 for drugs that follow Eq. 2, and these 5 parameter estimates, the interaction indices were calculated for the 25 combination points and listed in the tenth column of table 3. When $I > 1$, Loewe antagonism is claimed; when $I < 1$, Loewe synergism is claimed. The results of this analysis are listed in column 11 of table 3. There were 21 cases of Loewe synergism and 4 cases of Loewe antagonism. The overall conclusion is Loewe synergism, in agreement with the isobologram by hand method, but in apparent disagreement with the fractional product method of Webb (1963), the method of Valeriote and Lin (1975), and the method of Drewinko et al. (1976).

The advantages and disadvantages of the interaction index method of Berenbaum (1977) are similar to the isobologram by hand method. The key advantages include:

(*a*) the null reference model is the Loewe additivity model, Eq. 6.

(*b*) if the individual concentration-effect curves for both drugs can be well characterized, then all of the combination data can be used. This eliminates some of the potential waste of data of the isobologram by hand method. Also, in principle, the experimental designs can be parsimonious.

The key disadvantages include:

(*a*) it is not obvious how to derive a good summary measure of the intensity of interaction. If one merely calculates a mean for all of the *I*s and then performs a Student's t-test with the null hypothesis that the true interaction index is equal to 1, then the same criticisms directed against the mean Drewinko score would apply here.

(*b*) the analysis results are not as visually informative as with the isobologram by hand method.

F. Method of Steel and Peckham (1979)

This approach has many similarities to the isobologram by hand approach but also several fundamental differences. In addition to the original reference, the approach is described well by Streffer and Müller (1984) and by Calabrese (1991). A variant of the original approach developed by Deen and Williams (1979) has been used extensively by Teicher and coworkers (e.g., Teicher et al. 1991). First, reference curves for the Bliss independence model, Eq. 11 (called Mode I additivity) and for Mode II additivity, Eq. 20, are constructed for a particular effect level. An alternative equation for Mode II is provided by Kodell and Pounds (1991), although it is more common to describe the Mode II calculation with a diagram (e.g., Steel and Peckham, 1979; Streffer and Müller, 1984). Mode I and Mode II isobols for the 20% survival level are shown for the analysis of the common data set in figure 15. All calculations and graphs were made with pencil, graph paper, and French curve. However, automated curve fitting computer programs for the approach have been developed (Teicher et al., 1985). The data points are the ID_{80} values estimated from families of log-linear concentration-effect curves (not shown), not from the linear-log curves in figure 12. The positions of the ID_{80} points in figure 15 differ a little from the positions in figure 14 because of the differences in how the concentration-effect curves were drawn. Note that there are two Mode II isobols. Especially note that the Mode II isobols are not the same as the "classical" isobol simulated from the Loewe additivity model, Eq. 6. This is in direct contradiction to claims that the Mode II model and Loewe additivity are the same (Teicher et al., 1991). [This contradiction is the result of Steel and Peckham's (1979) misinterpretation of the first paper on isobolograms in English by Loewe (1953). Unfortunately, this key paper, Loewe (1953), was written with a cryptic mathematical notation and is difficult to interpret. It is a dramatic contrast to his lucid original paper on the subject, Loewe and Muischnek (1926), written in German.] The area between the Mode I and Mode II isobols is called the "envelope of additivity."

Because most of the ID_{80} points fall between the borders of the envelope of additivity, using either Mode II isobol for the upper boundary, the conclusion for the common data set would be additivity.

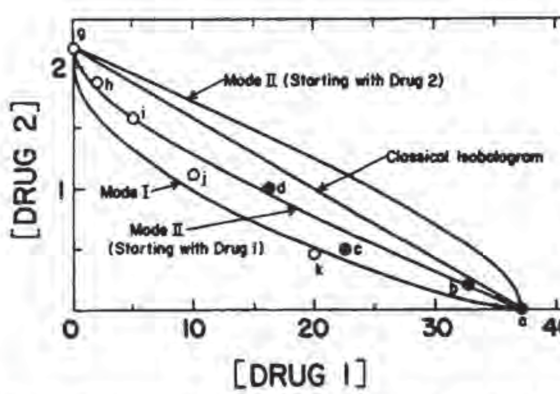


FIG. 15. Isobologram from the Method of Steel and Peckham (1979) for the 20% survival level (IC_{80}). Note that the isobol for the Mode I assumption, each of the two isobols for the Mode II assumption, and the isobol for the classical Loewe additivity assumption are all different. The data points are IC_{80}s taken from log-linear plots of %survival vs. drug concentration. The letters next to the points correspond to the legend of the linear-log survival plots in figure 13.

The advantages of the method of Steel and Peckham (1979) include:

(*a*) a region, the envelope of additivity, is provided to facilitate judgments about departures from no interaction, rather than a line. The envelope of additivity provides a standard, with a reasonable theoretical justification, to aid in the decision of whether a departure from additivity is great enough to warrant further consideration.

(*b*) the automated variant of the approach (Teicher et al., 1985) provides a degree of objectivity and some statistical rigor.

(*c*) the approach is widely accepted.

The disadvantages of the method include:

(*a*) neither of the two no interaction null reference models, that for Mode I or that for Mode II, are the preferred Loewe additivity model. The Mode II reference model is not part of other common approaches; in addition, it results in two predictions.

(*b*) the envelope of additivity does not take into account the precision of the data; it is not larger for data with more experimental error. It is not a statistical interval.

(*c*) the method lacks a summary measure of the intensity of interaction.

(*d*) the method is insensitive to small but real and potentially important interactions. It lacks good statistical power. This was seen for the analysis of the common data set.

G. Median-effect Method of Chou and Talalay (1984)

Of all of the methods examined in this paper, the median-effect approach received the most thorough review. This is because, of all of the methods to assess agent interaction introduced since 1970, the method of Chou and Talalay (1984) has been the most influential and controversial. Probably the key element of the approach that has led to its widespread use is the availability of an implementation in inexpensive microcomputer software (Chou and Chou, 1987). Chou (1991a) lists 79 recent publications that applied the median-effect approach to real laboratory data; 39 centered on anticancer agents, 25 centered on antiviral agents, and 15 centered on other miscellaneous agents. Our own literature survey located 3 application papers in 1985, 5 in 1986, 13 in 1987, 16 in 1988, 28 in 1989, 31 in 1990, and 11 in an incomplete survey of 1991 for a total of 107. It is clear that the approach has many advocates and that its use has continued to grow. The article, Chou and Talalay (1984), may become one of the most often-referenced scientific papers in the history of biomedicine.

The median-effect approach is the culmination of a long series of very technical papers centered on describing a wide variety of complex enzyme kinetic mechanisms with a general framework (see Chou, 1991a for a summary). Many useful concepts and equations were introduced by this series of papers, including several used by our group in the development of our own response surface approach for assessing agent combinations (Greco et al., 1990). In fact, our original motivation in developing our approach was merely to add small improvements to the median-effect method. For instance, our first goal was to show (via Monte-Carlo simulation) that using weighted nonlinear regression to fit a nonlinear form of the median-effect equation, Eq. 1, to single drug data was superior to using unweighted linear regression to fit a linearized form of the median-effect model, Eq. 23, to single drug data (Syracuse and Greco, 1986). Even though the weighted nonlinear regression approach was consistently more precise and less biased than the unweighted linear regression approach, for the estimation of both Dm and m, the differences were usually not striking, and the simpler method performed very well for most cases. However, as we examined the method of Chou and Talalay (1984) more closely, we found several disturbing problems, which will be described below. In addition, our own approach developed along very different lines, most notably with the incorporation of some ideas of Berenbaum (1985). Today, our approach for assessing agent interaction (Greco et al., 1990) bears only a faint resemblance to the median-effect method.

The analysis of the common data set by the approach of Chou and Talalay (1984) is shown in figure 16. Only a

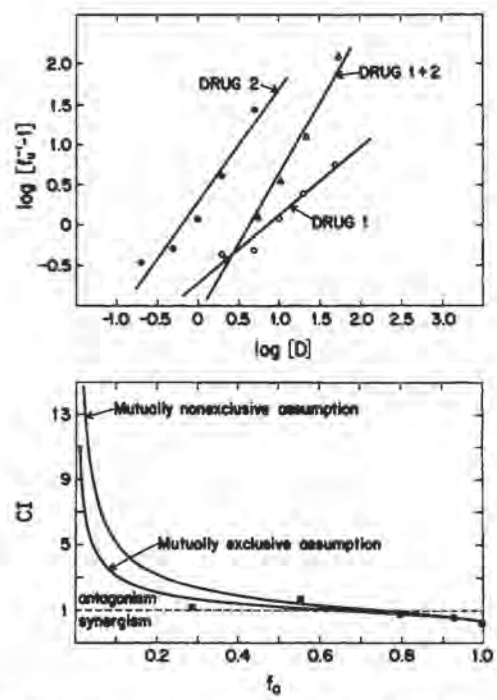


FIG. 16. Median-effect (upper panel) and CI vs. fa plot (lower panel) for the analysis of data from table 3, columns 2 through 4, for drug 1 alone (points 4 through 8), drug 2 alone (points 9 through 13) and for the combination at a fixed ratio of D_1:D_2 of 10:1 (points 14, 20, 26, 32, and 38) The solid square data points in the lower panel represent the five combination points and were calculated as described in the text.

brief description of the approach is included here; there have been many detailed recipes of the approach previously published (e.g., Chou and Talalay, 1984; Chou and Chou, 1987; Chou, 1991b; Calabresi, 1991). The easiest way to apply the approach to a data set is to use the software program by Chou and Chou (1987), which is available for both the Apple II and IBM-compatible personal computers. Eq. 23 is fit to data from drug 1 alone, drug 2 alone, and the combination of drug 1 and drug 2 in a fixed ratio. [Eq. 23 is a linearized form of Eq. 24, essentially equivalent to the Hill equation, Eq. 2, and was derived by Chou and Talalay (1981).]

$$\log[fu^{-1} - 1] = \log[fa^{-1} - 1]^{-1} = m\log(D) - m\log(Dm) \qquad [23]$$

$$\frac{fa}{fu} = \left(\frac{D}{Dm}\right)^{m} \qquad [24]$$

An average control effect was first calculated (the average of the 3 $D_1 = D_2 = 0$ points, 106, 99.2, and 115 from column 4 of table 3) to be 107. Then, each fu value was calculated by dividing the measured effect in column 4 by 107. For drug 1 alone, points 4 to 8 were used, for drug 2 alone, points 9 to 13 were used, and for the combination at a fixed ratio of 10:1, points 14, 20, 26, 32, and 38 were used. (In principle, more sets of points from other fixed ratios from the data set in table 2 could have been used for the analysis; however, it is very common to apply the approach to a single fixed ratio.) Additional calculations were performed on the 15 data points to construct the transformed y-values of $log[fu^{-1}-1]$ and the transformed x-values of $\log(D)$. Unweighted linear regression was applied separately to the three sets of five points each, and the slopes and y-intercepts were estimated, m and $-m\log(Dm)$, respectively. The transformed data and fitted curves are in the upper panel of figure 16. The Dm values were calculated from the y-intercepts and slopes. The six estimated parameters were: for drug 1, $Dm_1 = 7.40$, $m_1 = 0.845$; for drug 2, $Dm_2 = 0.631$, $m_2 = 1.37$; for drug 1 + 2 in a fixed ratio, $Dm_{12} = 4.48$ and $m_{12} = 1.77$. [Note that the signs of the ms have been made positive to correspond to the standard implementation of the approach of Chou and Talalay (1984); this is the opposite of the convention usually used by our group.] According to Chou and Talalay (1984), if $m_1 = m_2 = m_{12}$, then the two drugs are claimed to be mutually exclusive; if $m_1 = m_2 \neq m_{12}$, then the two drugs are claimed to be mutually nonexclusive; if $m_1 \neq m_2$, the mutual exclusivity of the drugs is unclear. Chou and Talalay (1984) do not explicitly state how the equivalencies of m_1, m_2, and m_{12} should be determined. However, we will make the conclusion that 0.845, 1.37, and 1.77 are sufficiently different from each other that the mutual exclusivity is unclear for the common data set.

In the lower panel of figure 16 are the CI vs. fa plots for both the mutually exclusive and mutually nonexclusive assumptions. These plots were generated by inserting the six estimated parameters from the median effect plots into Eq. 25 for the mutually exclusive case and into Eq. 26 for the mutually nonexclusive case (Chou and Chou, 1987; Chou, 1991b), and calculating CI for the range of fa from 0.01 to 0.99. (Here, R is the ratio of concentrations of D_1:D_2). The area above the $CI = 1$ line represents antagonism; below, synergism. The five data points in the lower panel represent the five combination points that have been transformed with Eq. 27 and directly plotted, without relying on the estimation of Dm_{12} and m_{12}. This addendum to the approach, suggested mainly for nonconstant combination ratios (Chou, 1991a), is also applicable to fixed combination ratios, as shown by our example. To the best of our knowledge, it is not yet available in the commercial software (as of August, 1992). This is essentially the same approach as described in Section V.E., the calculation of Berenbaum's (1977) interaction index.

$$CI = \frac{Dm_{12}\left[\frac{R}{R+1}\right]\left[\frac{fa}{1-fa}\right]^{1/m_{12}}}{Dm_1\left[\frac{fa}{1-fa}\right]^{1/m_1}} + \frac{Dm_{12}\left[\frac{1}{R+1}\right]\left[\frac{fa}{1-fa}\right]^{1/m_{12}}}{Dm_2\left[\frac{fa}{1-fa}\right]^{1/m_2}} \qquad [25]$$

$$CI = \frac{Dm_{12}\left[\frac{R}{R+1}\right]\left[\frac{fa}{1-fa}\right]^{1/m_{12}}}{Dm_1\left[\frac{fa}{1-fa}\right]^{1/m_1}} + \frac{Dm_{12}\left[\frac{1}{R+1}\right]\left[\frac{fa}{1-fa}\right]^{1/m_{12}}}{Dm_2\left[\frac{fa}{1-fa}\right]^{1/m_2}} + \frac{Dm_{12}^2\left[\frac{R}{(R+1)^2}\right]\left[\frac{fa}{1-fa}\right]^{2/m_{12}}}{Dm_1 Dm_2\left[\frac{fa}{1-fa}\right]^{1/m_1}\left[\frac{fa}{1-fa}\right]^{1/m_2}} \qquad [26]$$

$$CI = \frac{D_1}{Dm_1\left[\frac{fa}{1-fa}\right]^{1/m_1}} + \frac{D_2}{Dm_2\left[\frac{fa}{1-fa}\right]^{1/m_2}} \qquad [27]$$

Overall, the conclusion is strong antagonism at low fas, slight synergism at $fa > 0.8$, with the assumption of mutual nonexclusivity; strong Loewe antagonism at low fas, slight Loewe synergism at $fa > 0.8$, with the assumption of mutual exclusivity. Note that the extreme antagonism occurs to the left of the combination data points. If one would just examine the five combination points calculated with Eq. 27, then one might conclude Loewe additivity; or slight Loewe antagonism at low fas and slight Loewe synergism at high fas.

The advantages and good features of the median-effect approach of Chou and Talalay (1984) include:

(*a*) the fundamental equations for the approach were derived from basic mass action enzyme kinetics, and thus, the estimable parameters have the potential to be biologically meaningful. However, the approach has most often been applied to much more complex systems, such as biochemical networks, viruses, bacterial cells, mammalian cells, intact mammals, or populations of mammals. Therefore, the biochemical origin of the median-effect approach, a relatively simple system of multiple inhibitors of a single enzyme, will usually not facilitate mechanistic insights into the more complex systems to which the approach is applied. The mechanistic models of the approach are used essentially in an empirical manner.

(*b*) many useful equations, combined-action concepts, and specific applications of the approach have been published that have inspired others to create newer approaches (e.g., Greco et al. 1990).

(*c*) part of the method involves the fitting of models to data with an objective, well accepted statistical approach, namely linear regression.

(*d*) the experimental design requires fewer data points than a typical design to be analyzed by the isobologram technique and other methods. However, the common sparse design with one fixed ratio of D_1:D_2 may miss some interesting regions of the full 3-D concentration-effect surface (Prichard and Shipman, 1990).

(*e*) the mutually exclusive model is consistent with the Loewe additivity null reference model.

(*f*) for many analyses of real data, when artifacts inherent in the approach do not make a major contribution, the overall general conclusions will be consistent with more rigorous methods. However, conversely, when artifacts do make a major contribution, the final conclusions will not be consistent with more rigorous methods. For example, in an informal survey of 37 application papers that used the Chou and Talalay (1984) approach, we re-analyzed 136 data sets with the parametric model fitting approach, using Eq. 5, described in Section V.L.1. For only 38 of the 136 data sets (28%) was there close agreement in the final conclusions for the two approaches.

(*g*) the method is available in microcomputer software for the popular Apple II (Apple Computer Inc., Cupertino, CA) and IBM PC (IBM Corporation, Boca Raton, FL) (and compatible) microcomputers. This last advantage is the most crucial: for any sophisticated data analysis technique to be used routinely by biomedical scientists, especially by those with little mathematical and statistical training, the method must be readily available in the form of inexpensive, user-friendly software.

The disadvantages of the method of Chou and Talalay (1984) include:

(*a*) the mutually nonexclusive model was not adequately derived. Appendix A includes an extensive discussion of this point, provides a derivation from basic enzyme kinetic arguments for Eq. 12, a model that can also be derived directly from the concept of Bliss independence, and provides support for Eq. 12 being a more appropriate model for mutual nonexclusivity for two inhibitors against a single enzyme, than Chou and Talalay's model 18 (or an alternate form, Eq. 19). It must be noted that, as shown in Appendix A, the mutually nonexclusive model of Chou and Talalay (1984) for two inhibitors of a single enzyme can be derived from enzyme kinetic arguments by making some additional assumptions. However, it is unlikely that an equation derived from a set of unusual assumptions, for a rare experimental system, would have general utility for modeling concentration-effect phenomena from a wide spectrum of complex agent interaction systems. Another implication of this discussion is the weakness of Chou and Talalay's (1984) argument that the fractional product method of Webb (1963) is not valid for higher order systems with sigmoidal concentration-effect curves ($|m| > 1$). In fact, from a theoretical basis, any approach based upon Loewe additivity or Bliss independence is "valid" for most types of concentration-effect functions over a wide range of parameter values.

(*b*) as shown in Appendix B, Nonlinear Nature of the Median Effect Plot for Mutual Nonexclusivity section, the median-effect plot for mutually nonexclusive inhibitors is not linear; this leads to inaccuracies in the estimation of Dm_{12} and especially of m_{12} via linear regression, and then to artifacts in the *CI* vs. *fa* plot, including large antagonism at low *fa*s. Interestingly, this nonlinearity in the median-effect plot for their mutually nonexclusive model was first shown by Chou and Talalay (1981) in their figure 2 (not shown here).

(*c*) the *CI* formula for the mutually nonexclusive case is not correct. This is shown in Appendix B, Incorrect Combination Index Calculations for the Mutually Nonexclusive Case section. This also leads to artifacts in the *CI* vs. *fa* plot.

(*d*) even for the mutually exclusive case, one effect of Loewe synergism or Loewe antagonism is to make the median-effect plot nonlinear, leading to artifacts in the *CI* vs. *fa* plot. This is shown in Appendix B, Nonlinear Nature of the Median Effect Plot for Mutual Exclusivity with Interaction section.

(*e*) The median-effect equations for both the mutually exclusive and nonexclusive cases were originally derived by Chou and Talalay (1981) with the assumption that $m_1 = m_2$. When $m_1 \neq m_2$, which is usually the case, both models are only approximately valid. The approximation becomes worse as the difference between the *m*s becomes larger. This problem and several others are illustrated in figure 17. Eight simulations were conducted using Eq. 5 as *a* model (not *the* model) for Loewe synergism or Loewe antagonism, using the values for m_1, m_2 and α listed in the insets of the figure. The simulated data were plotted in panel A after the median-effect transformation. The *CI* vs. *fa* plots were simulated directly with Eq. 8, thus avoiding many of the calculation

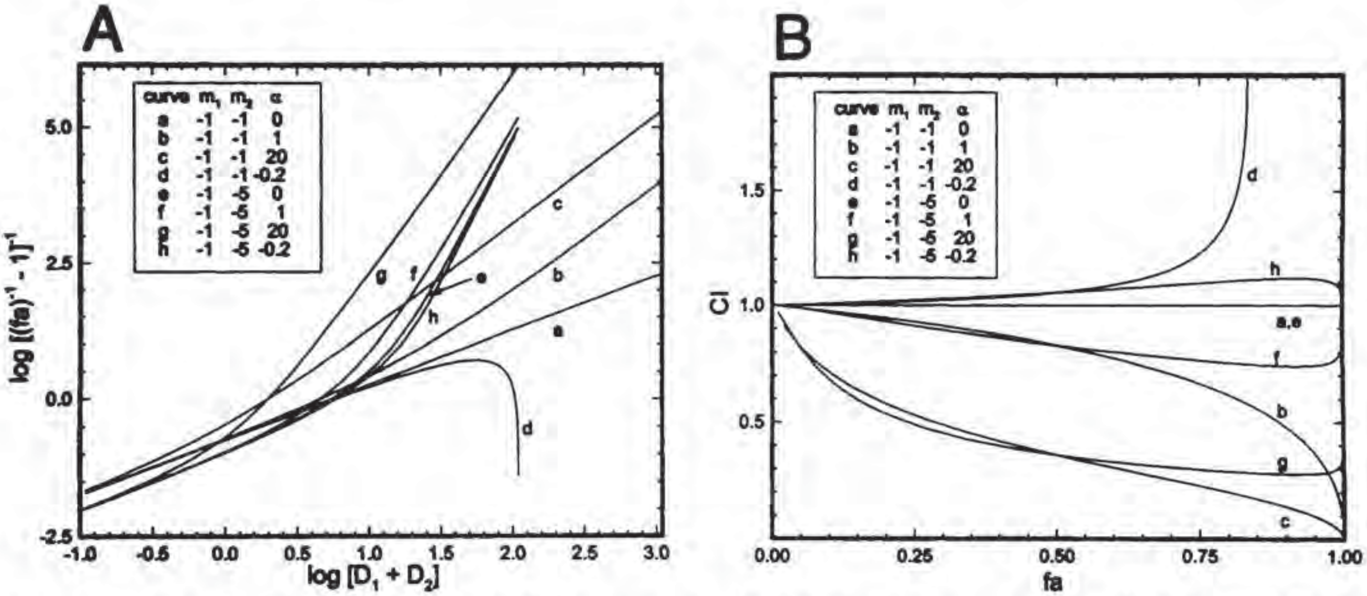


FIG. 17. Median-effect plots (*A*) and *CI* vs. *fa* plots (*B*) for data simulated with Eq. 5, with parameters: $Econ = 100$, $IC_{50,1} = 10$, $IC_{50,2} = 1$ and m_1, m_2, α as listed in the inset boxes in each panel. *CI* was calculated from Eq. 8. Note that the median-effect plot is a straight line only for the case in which $m_1 = m_2$ and $\alpha = 0$. Thus, both $m_1 \neq m_2$ and $\alpha \neq 0$ will result in a curved median-effect plot. Also note that the shape of the *CI* vs. *fa* plots are influenced by both the slope parameters and the interaction parameter.

artifacts discussed in points (*b*) through (*d*) of this section. Note that the median-effect plot is a straight line only for the case a., in which $m_1 = m_2 = -1$, and $\alpha = 0$. Thus, either $m_1 \neq m_2$, or $\alpha \neq 0$, or both conditions will result in a curved median-effect plot. Note that large differences in slope parameters (e.g., curve *e.*, $m_1 = -1$, $m_2 = -5$, $\alpha = 0$) seem to have a more profound effect on the curvature than does a high α value (e.g., curve *c.*, $m_1 = -1$, $m_2 = -1$, $\alpha = 20$). Because only pure Loewe additivity, pure Loewe synergism, or pure Loewe antagonism were simulated, none of the *CI* vs. *fa* plots cross the $CI = 1$ line. Note that all of the plots, for both Loewe synergism and Loewe antagonism, start at $CI = 1$ ($fa = 0$). This implies that all reported *CI* vs. *fa* plots that show large antagonism in the region near $fa = 0$, contain calculation artifacts. Indeed, the $CI = 1$ at $fa = 0$ point should be the anchor for all *CI* vs. *fa* plots, no matter what kind of combined-action is present. Also note that *CI* vs. *fa* curves *b.* and *c.* ($m_1 = m_2 = -1$) curve downward near $fa = 1$, whereas, curves *f.* and *g.* ($m_1 = -1$, $m_2 = -5$) curve upward near $fa = 1$. Finally, note that increasing degrees of Loewe synergism, for the same set of slope parameters, order the curves from bottom to top for the median-effect plot, but from top to bottom for the *CI* vs. *fa* plot. It is clear that in the vast majority of cases, the median-effect linearization of combination data at a fixed ratio will result in a true nonlinear curve. The nonlinearity may be small, and data variation may mask the nonlinearity, but the fitting of a median-effect straight line to such data will almost always be, at best, only approximately correct.

(*f*) the method of Chou and Talalay (1984) lacks many aspects of modern statistical approaches. First, the fitting of the median-effect line to data with linear regression does not have the option of weighting. However, proper weighting only offers a slight improvement to the unweighted linear regression (Syracuse and Greco, 1986). Second, the only goodness of fit statistics offered are Pearson correlation coefficients, *r*, for each separate unweighted linear regression of the transformed data for each median-effect plot. It would be useful to have some overall goodness of fit statistic for the fit of the overall model simultaneously to all of the data. There is no uncertainty measure provided with the estimates of m_1, m_2, and m_{12} to aid in making the decision between mutual exclusivity vs. mutual nonexclusivity. Most importantly, there is no uncertainty measure associated with the final result, the *CI* vs. *fa* plot. Objective decisions regarding the occurrence of moderate degrees of Loewe synergism or Loewe antagonism are therefore difficult. However, newer variants of the approach include more extensive statistical procedures, such as confidence intervals for the combination index (Belen'kii and Schinazi, 1994).

(*g*) the relationship between the *CI* vs. *fa* plot, the original raw data, and the original concentration-effect curves is somewhat hard to visualize. The experimenter may "lose touch" with his data. However, a good understanding of the relationship between the *CI* vs. *fa* plot and the 3-D concentration-effect surface for a two drug combination, figure 7, may assist in this visualization.

(*h*) the Chou and Talalay (1984) approach first involves a decision on mutual exclusivity vs. mutual nonexclusivity, and then a decision on synergism, additivity, or antagonism, for a total of six different cases. There is a conceptual difficulty in differentiating between mutual exclusivity with synergism and mutual nonexclusivity with synergism, additivity, and especially with antagonism. The regions overlap. This can be seen in isobols of figure 6, in which curve E represents pure mutual exclusivity ($\alpha = 0$), curve C represents pure mutual nonexclusivity ($\alpha = 1$), and curve D ($\alpha = 0.5$) would be an example of Loewe synergism with reference to the mutually exclusive model and of Loewe antagonism with reference to the mutually nonexclusive model. In line with this reasoning, the figure legend of figure 2 from Chou and Talalay (1981) states that the curve for mutual nonexclusivity "clearly shows synergistic effects at high concentrations. . . . " In fact, one can see that the nonlinear form of the mutually nonexclusive model, Eq. 19, is the same as our flagship model for Loewe synergism, Eq. 5, with $m = m_1 = m_2$ and $\alpha = 1$.

(*i*) the available software (Chou and Chou, 1987) that implements the approach is relatively unsophisticated. Future changes in the computer software should include improvements in graphics, datafile editing, saving and retrieving, and the prevention of the program from "bombing" under certain conditions.

(*j*) if the concentration-effect curve for either agent in a combination does not follow the Hill model, Eq. 1 (or the equivalent median-effect model, Eq. 24), then the Chou and Talalay (1984) approach is not valid.

(*k*) there are three practical decisions that users of the Chou and Talalay (1984) approach must make that critically affect the final results: (1) what to do with data points in which % survival equals or exceeds 100%, or equals or is less than 0%; such data will lead to computational difficulties; (2) how to decide whether a specific two-agent interaction is mutually exclusive or mutually nonexclusive, especially when $m_1 \neq m_2$; and (3) how to conclude synergism, additivity, or antagonism from the *CI* vs. *fa* plot. There is a wide variety of different tactics used by different groups to make these three critical decisions. Therefore, the objectivity of the approach is lessened. For example, for decision (1), some groups either censor any extreme points ($fa \geq 1$, $fa \leq 0$) or change any $fa \geq 1$ to a usable *fa* such as 0.96 (e.g., Schinazi et al., 1986), whereas, most groups do not specify their procedure (e.g., Hartshorn et al., 1986). For decision (2), as recommended by Chou and Talalay (1984), some assume mutual exclusivity when the median-effect plots for both single drugs and the combination are parallel (e.g., Koshida et al., 1989), assume mutual nonexclusivity when the slope parameters for the single drugs are similar but the slope for the combination is much different (e.g., Nocentini et al., 1990), and report both exclusivities when the median-effect plots for both single drugs are not parallel (e.g., Eriksson and Schinazi, 1989). However, some groups report the mutual exclusivity results, because they feel that the mutually nonexclusive results would not be much different (e.g., Vogt et al., 1987; Kuebler et al., 1990). Some report mutual exclusivity, because it corresponds to the classical isobologram approach (e.g., Johnson et al., 1992); some groups assume mutual nonexclusivity, because it yields a more conservative estimate of *CI* (e.g., Vathsala et al., 1990). Some assume mutual nonexclusivity, because the two agents are known to act at different sites (e.g., Jackson, 1992), and some assume some exclusivity, but don't state which one or why (e.g., Richman et al., 1991). For decision (3), some groups stress the *CI* at high *fa*s, such as 0.50, 0.75, 0.90 and 0.95 (e.g., Kong et al., 1991). Some show the whole *CI* vs. *fa* plot, from 0.01 to 0.99 and describe many of the nuances of the curve, including the point at which the $CI = 1$ line is crossed (e.g., Wadler et al., 1990). Some report an average *CI* for the 50% effect point from several replicate experiments, along with a standard deviation (e.g., Katz et al., 1990). Some use several other additional approaches to analyze the data, such as the isobologram approach, or the method of Steel and Peckham (1979) and then report a consensus (e.g., Nocentini et al., 1990). There are no firm guidelines for assessing the importance of small consistent differences between the *CI* vs. *fa* plot and the $CI = 1$ line. For example, in Chou and Chou (1987), the *CI* vs. *fa* plot on page 42 follows a path slightly above the $CI = 1$ line, with a conclusion of additivity; whereas, the *CI* vs. *fa* plot on page 61 follows a path slightly below the $CI = 1$ line, with a conclusion of strong synergism.

H. Method of Berenbaum (1985)

In one sense, the method of Berenbaum (1985) is merely a graphical version of the interaction index approach of Berenbaum (1977). However, interpreted differently, the method of Berenbaum (1985) is the basis of all modern nonparametric and parametric response surface approaches to be described in Sections V.K. and V.L. The approach consists of fitting concentration-effect models to data for each agent alone, deriving a model for Loewe additivity consistent with these single agent models, simulating the Loewe additivity model, superimposing this simulated Loewe additivity surface upon the raw data points, and then deciding whether points are above or below the surface, which will indicate Loewe synergism or Loewe antagonism, depending upon whether the 3-D concentration-effect surface rises or falls with increasing agent concentrations. The derived Loewe additivity models can accommodate different slope parameters for each agent when each agent's concentration-effect curve follows a Hill model, Eq. 2, 3. The Loewe additivity models can even accommodate different functional forms for the concentration-effect curve for each agent. Unfortunately, these models are often in unclosed form. A formal parametric model for Loewe additivity is useful, but optional: Berenbaum (1985) shows an example of fitting complex single agent data by hand. Sühnel (1992c) has derived and listed many parametric Loewe additivity models and emphasizes the use of 3-D interaction plots, such as figure 9, and 3-D difference surfaces such as in figure 10. The functional form of the derived Loewe additivity response surfaces, e.g., Eq. 13, can easily be extended to include interaction terms, leading to a full combined-action model, such as Eq. 5. In fact, the guidelines from Berenbaum (1985) for deriving general Loewe additivity models led us directly to the derivation of Eq. 5, which was first published in Syracuse and Greco (1986). Interestingly, essentially the same logic for deriving Loewe additivity and combined-action models was part of a review paper by Hewlett (1969), who provides examples of combined-action models from Finney (1952), Plackett and Hewlett (1952), Landahl (1958), and Plackett and Hewlett (1967). However, Berenbaum's (1985) hallmark paper is much clearer and was published at a time when the necessary computer hardware and software were sufficiently available to enable the routine application of his paradigm and logical variants to real data.

We applied the method of Berenbaum (1985) to the common data set by first fitting the first 13 data points in columns 2 to 4 of table 3 with Eq. 13, that for Loewe additivity for two inhibitory drugs that both individually follow Eq. 2, just as described for the interaction index approach of Berenbaum (1977) in Section V.E. The first 13 data points include the control points plus the drug 1 alone and drug 2 alone points. Just as in Section V.E., data were fit with nonlinear regression, weighted by the reciprocal of the square of the predicted effect. The five parameter estimates were: $Econ = 99.2 \pm 5.2$; $IC_{50,1} = 9.52 \pm 1.7$; $IC_{50,2} = 0.966 \pm 0.094$; $m_1 = -0.989 \pm 0.11$; $m_2 = -1.93 \pm 0.13$. Then, instead of calculating an interaction index using Eq. 8, the fitted curve is shown in figure 18(A), along with the raw data. For the 25 combination points, a solid point (above the surface) indicates Loewe antagonism, and an open point indicates Loewe synergism. The results are identical (as they must be) to the results from the interaction index approach of Berenbaum (1977) shown in columns 9 to 11 of table 3. There were 21 cases of Loewe synergism and 4

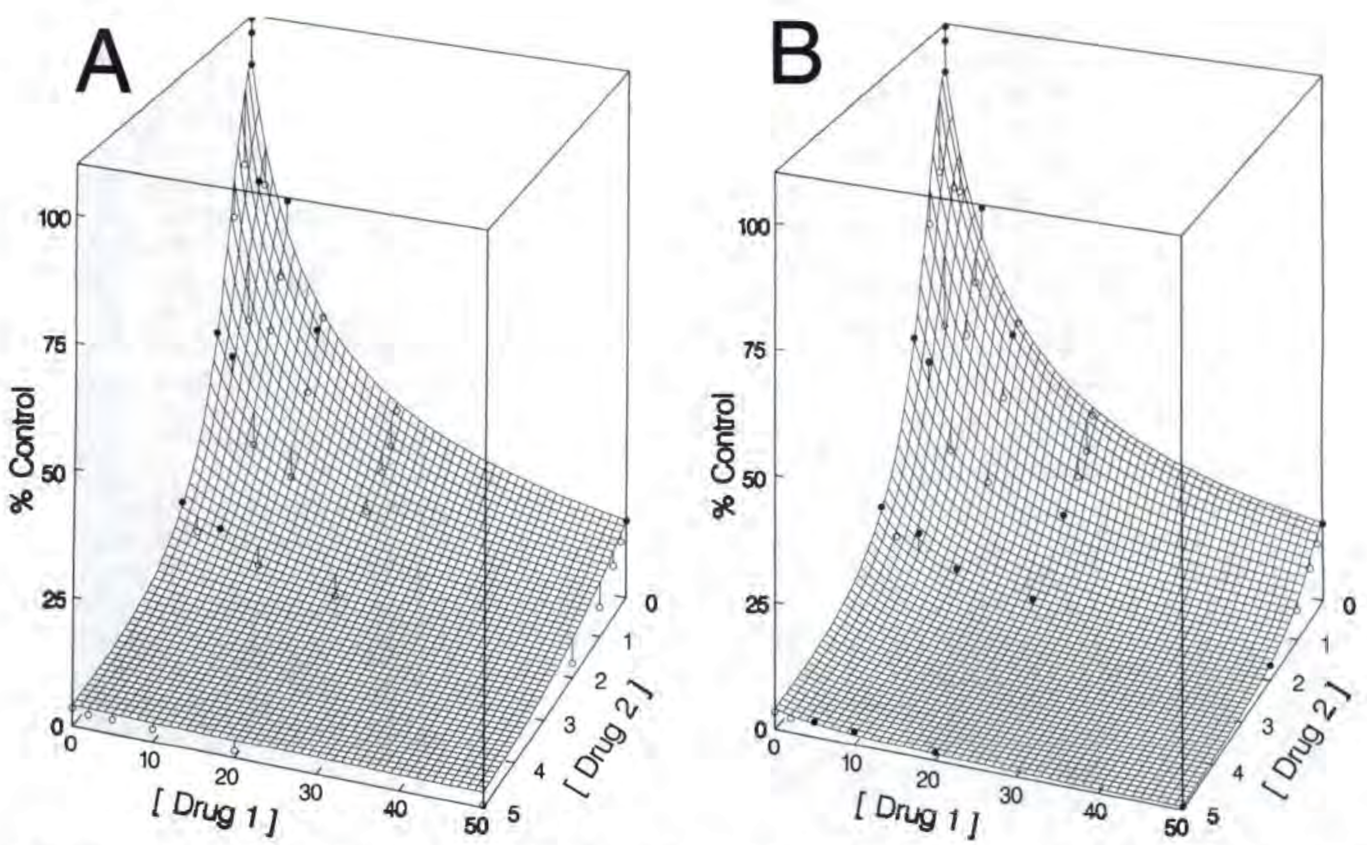


FIG. 18. Analyses of data from table 3, columns 2 through 4. (*A*) Approach interpreted from Berenbaum (1985). Data for drug 1 alone and drug 2 alone were fit by a Loewe additivity model, Eq. 13, with nonlinear regression as explained in the text. The 3-D fishnet is the best fit Loewe additivity surface. The full 38-point data set is plotted on the same graph, with vertical lines indicating the distance between the data points and the surface. Solid points are above the surface, and open points are below. For the 25 combination points, a solid point indicates Loewe antagonism, and an open point, Loewe synergism. There is an exact correspondence between this 3-D graph and columns 9 through 11 of table 3. (*B*) Graphical Bliss independence comparison. The best fit parameters from the fit of the Loewe additivity model, Eq. 13, were estimated as for panel (*A*), but these parameters were used with the Bliss independence model, Eq. 12 to simulate the 3-D surface. The full 38-point data set is again plotted on the same graph. There are 11 points above the surface (Bliss antagonism), and 14 points below the surface (Bliss synergism).

cases of Loewe antagonism. The overall conclusion is Loewe synergism.

The key advantages include:

(*a*) the null reference model is the Loewe additivity model, Eq. 6.

(*b*) if the individual concentration-effect curves for both drugs can be well characterized, then all of the combination data can be used.

(*c*) the experimental designs can be parsimonious.

(*d*) the single agent data are fit with a logical response surface model, possibly with modern curve fitting techniques.

(*e*) it is not necessary to derive or use some arbitrary combined-action model for fitting the combination data. Mosaics of regions of Loewe synergism and Loewe antagonism are thus easily accommodated.

(*f*) the approach led to the creation and use of full combined-action models (e.g., Greco et al., 1990).

(*g*) the approach can be used to characterize very complex mixtures of three or more agents. If one is chiefly interested in the assessment of combined-action at a specific combination of doses of the agents and not in characterizing the whole response surface, then experimental designs can be very frugal.

The key disadvantages include:

(*a*) just as with the interaction index calculation approach (Berenbaum, 1977), it is not obvious how to derive a good summary measure of the intensity of interaction, with an accompanying measure of uncertainty. However, Gennings (1995) recently proposed some extensions to Berenbaum's (1985) method that include some excellent statistical summary measures of departures from Loewe additivity.

(*b*) the derivation and application of complex Loewe additivity models may require considerable mathematical, statistical, and computing resources.

I. Bliss (1939) Independence Response Surface Approach

We did not find this specific method in the literature, but it is included because it is a logical cross between the Webb (1963) and Berenbaum (1985) approaches. This approach is a graphical version of the fractional product method of Webb (1963) and is similar, but not identical, to the method of Prichard and Shipman (1990) described in Section V.J. The results are shown in figure 18(B), which was made in the same way as described in Section V.H. for the Berenbaum (1985) approach, except that the Bliss independence model, Eq. 12, was used to simulate the 3-D surface. There are 11 points above the surface (Bliss antagonism) and 14 points below the surface (Bliss synergism). The overall conclusion would be Bliss independence. Interestingly, the results differ from those previously found with the fractional product approach (Webb, 1963) (column 6 of table 2; 4 cases of Bliss synergism and 21 cases of Bliss antagonism). This difference is caused by the use of fitted individual concentration-effect curves for making the Bliss independence predictions for the surface approach, vs. the raw data for the individual drugs for making the Bliss independence predictions for the fractional product method.

This approach shares advantages (*b*) through (*e*) of the Berenbaum (1985) approach. It is possible that full combined-action models can be derived and applied, as suggested by Unkelbach (1992).

The key disadvantages include:

(*a*) the basis of the approach is Bliss independence, not our Loewe additivity preference.

(*b*) it is not obvious how to derive a good summary measure of the intensity of interaction, with an accompanying measure of uncertainty. However, variants of the recently proposed extensions by Gennings (1995) to Berenbaum's (1985) approach may solve this problem.

(*c*) the derivation and application of complex Bliss independence models may require considerable mathematical, statistical, and computing resources.

J. Method of Prichard and Shipman (1990)

This approach (e.g., Prichard et al., 1990) is a graphical, 3-D version of the fractional product method of Webb (1963). Figure 19 shows the result of the analysis of the common data set, columns 2 through 4 of table 3. A checkerboard (factorial) experimental design, like that provided by the common data set, is necessary for the optimal use of the approach. We used the MacSynergy II program (Prichard et al., 1992), which is a set of MicroSoft Excel (Microsoft Corporation, Redmond, WA) spreadsheets and macros, kindly provided by M. Prichard, which was run with Excel to perform the necessary calculations. We used the Tecplot graphics package (Amtec Engineering, Inc., 1988) to prepare figures 19 and 20.

First, the % inhibition for every data point is calculated (100% − column 3 of table 3 divided by the average control, 106.7). (Note that 107 was the average control value used to generate columns 5 and 8 in table 3.) The points, connected with straight lines, are plotted on a 3-D graph in figure 19(A). The predictions, based upon Bliss independence, are calculated on a point-by-point basis, just as with the Webb (1963) approach and are plotted in figure 19(B). Figure 19(C) is the difference plot of the % inhibition above predicted. These differences are equivalent to the Drewinko et al. (1976) Scores in column 8 of table 3, after reversing the signs, and dividing the Drewinko Scores by the average control. There are 22 combination points below the zero plane, representing Bliss antagonism, and 3 points above the zero plane, representing Bliss synergism. These 3 data points are the same ones that showed Bliss synergism in table 3, data points 21, 26, and 36. The Bliss synergy differences were added up to yield a summary measure, 7.19, and the Bliss antagonism differences were added up to yield a Bliss antagonism summary measure, -65.27.

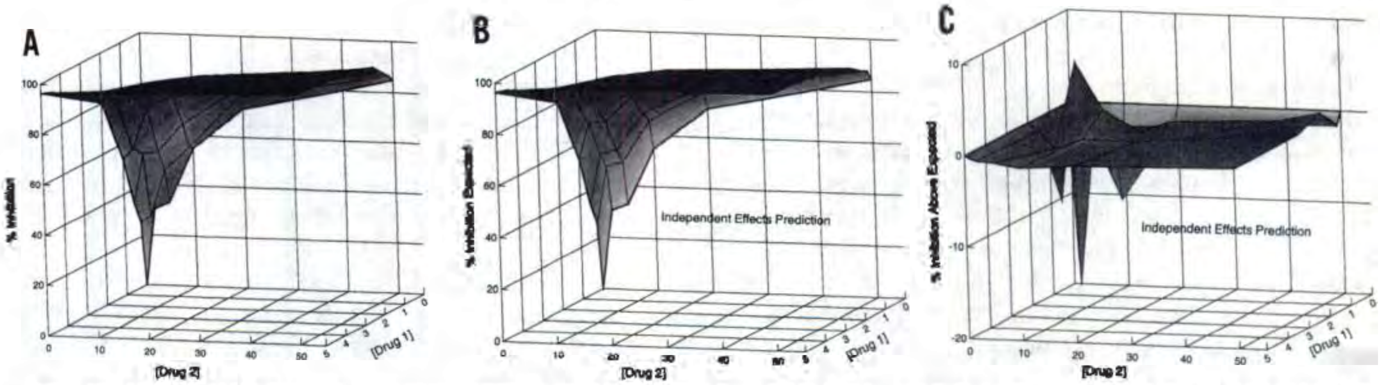


FIG. 19. Method of Prichard and Shipman (1990) applied to the data from table 3, columns 2 through 4. (*A*) Raw data, 36 data points (the 3 control points were averaged into 1 point), expressed as %inhibition, connected by straight lines, in a 3-D plot. (*B*) combination points are predicted directly from the raw data for drug 1 alone and drug 2 alone, with Eq. 11, that for Bliss independence, expressed as %inhibition, and connected with straight lines, in a 3-D plot. (*C*) The set of points from panel (*B*) are subtracted from the set of points from panel (*A*) and shown in a 3-D plot. Sections of the difference surface above 0 indicate Bliss synergism, below 0, Bliss antagonism. Both Bliss synergism and Bliss antagonism are seen.

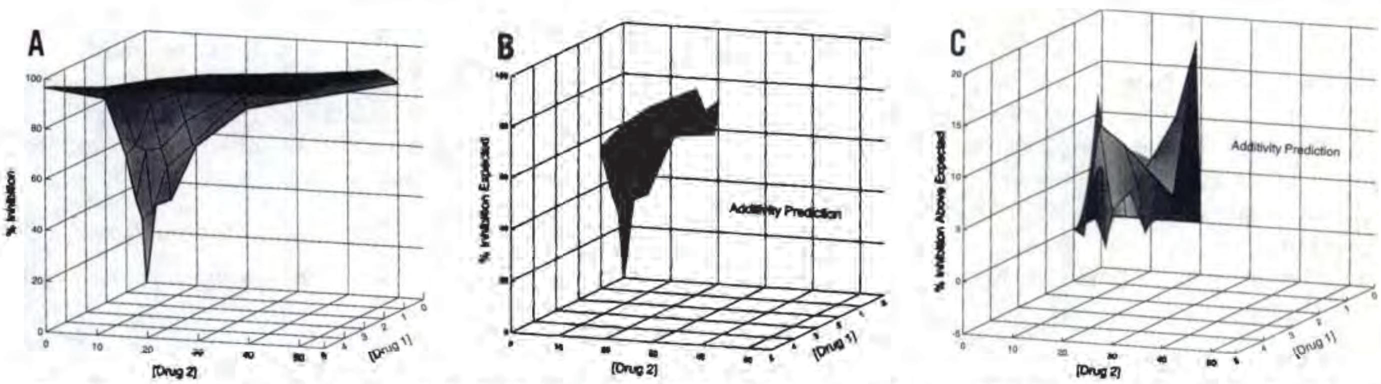


FIG. 20. An alternate approach provided by Prichard et al. (1992) that integrates the Loewe additivity reference concept of Berenbaum (1985), applied to the data from table 3, columns 2 through 4. (*A*) Same as panel (*A*), figure 19. (*B*) Predicted Loewe additivity surface analogous to panel (*B*) of figure 19. (*C*) Difference surface analogous to panel (*C*) of figure 19. Mostly, Loewe synergism is seen. The algorithm used by Prichard et al. (1992) does not make Loewe additivity predictions for points along the outer edge, and thus the predicted and difference surfaces appear to be smaller than those of figure 19.

Although we were able to successfully apply the Prichard and Shipman (1990) method to our common data set, the ideal data set for this approach will contain replicates. Replicates allow the calculation of point-by-point 95%, 99%, and 99.9% confidence intervals for the experimental data. If the lower confidence limit for a point is greater than the predicted Bliss independence, the observed Bliss synergy is considered to be significant. Similarly, if the upper confidence limit for a point is less than the predicted Bliss independence, the observed Bliss antagonism is considered to be significant. The significant Bliss synergism and antagonism differences are totaled separately for additional summary measures. The overall conclusion for the results of the analysis of our common data set is Bliss antagonism. However, as stated above, replicates are needed in order to make firm conclusions with this approach.

The main advantages of the approach are:

(*a*) the approach emphasizes the 3-D nature of combined-action concentration-effect surfaces; it is very visually oriented.

(*b*) the software, MacSynergy II, is inexpensive and straightforward to use, provided that one already is proficient with Excel (or possibly some other spreadsheet software) and a suitable graphics package.

(*c*) the approach is very flexible and does not require a parametric model for either the single agent concentration-effect curves or for combined-action. The approach is, essentially, a very simple nonparametric multivariate curve fitting procedure. The approach can easily accommodate mosaics of interspersed regions of Bliss synergism and Bliss antagonism.

(*d*) there are some summary and uncertainty measures associated with claims of Bliss synergism and Bliss antagonism.

(*e*) mathematical, statistical, and computing complexities associated with the fitting of full combined-action response surface models are avoided.

(*f*) when compared with all of the simpler approaches examined in this review, Sections V. A–V. G, the method of Prichard and Shipman (1990) stands out as having the best combination of automation, accessibility, intuitiveness, and visualization.

The disadvantages include:

(*a*) Bliss independence is the main no interaction reference model. However, a new feature added to MacSynergy II, but not necessarily recommended by Prichard et al. (1992), is the ability to use Loewe additivity as the null reference model. The results of the analysis of the common data set are displayed in figure 20. Note that the algorithm used by Prichard et al. (1992) does not make Loewe additivity predictions for points along the outer edge, and thus the predicted and difference surfaces appear to be smaller than those of figure 19. The conclusion for the analysis in figure 20 is Loewe synergism.

(*b*) the ideal experimental design, a full checkerboard of drug dilutions with replicates, may be prohibitive for many applications. However, for many in vitro studies of antiviral or anticancer agents, experimental systems use 96-well culture plates, which facilitates the requirement of a large experimental design.

(*c*) similar methods described in Sections V.H. and V.I., in which the data for drug 1 alone and drug 2 alone are fit by specific parametric models, but in which the combination points are not fit by specific combined-action models, may offer a cost-effective advantage over the Prichard and Shipman (1990) approach.

(*d*) the approach is essentially, an exploratory approach. It may be ideal as a front-end for further parametric 3-D response surface approaches for most data sets, or possibly a reasonable final method for very complex data sets with numerous regions of true Bliss synergism and Bliss antagonism. However, it might be of interest to test whether some of the mosaics of Bliss synergism and Bliss antagonism disappear after substituting Loewe additivity for Bliss independence as the no interaction null reference model. Data sets generated with a full replicated checkerboard design likely contain much more useful information than can be revealed by a simple exploratory approach. It would be cost-effective to further analyze such data sets with powerful multivariate parametric response surface approaches, such as described in Section V.L.

The paper that introduced the method of Prichard and Shipman (1990) also provided an extensive review of other older rival approaches. There were many confusing arguments included in this review, and because it may have had a large impact on workers in the antiviral chemotherapy field, and many of their arguments are at odds with our own views, some of Prichard and Shipman's (1990) assertions will be disputed:

(*a*) they claim that Chou and Talalay's (1984) mutually exclusive model is not equivalent to the Loewe additivity model. As shown in discussions of figures 7 and 8, and elsewhere in our review, they are indeed equivalent. Prichard and Shipman's (1990) assertion was based upon the unreasonable assumption of linear single agent concentration-effect curves, rather than sigmoidal curves following the Hill equation, Eq. 1.

(*b*) they claim that Loewe additivity is equivalent to fractional effect addition, Eq. 17, and to Steel and Peckham's (1979) Mode II model. All three models are different, as discussed in Section IV of our review. The cryptic paper of Loewe (1953) may be responsible for this confusion.

(*c*) they imply that Chou and Talalay's (1984) mutually nonexclusive model is, in general, equivalent to Bliss independence (Webb's 1963 model). This was shown not to be true in Appendix A and not to be true originally by Chou and Talalay (1984). Prichard and Shipman (1990) only examine the case of a first order system, an exceptional case in which the models are equivalent, as first demonstrated by Chou and Talalay (1984).

(*d*) Prichard and Shipman (1992) assert that the methods proposed by Sühnel (1990) and Greco et al. (1990) are not quantitative and that the method of Prichard and Shipman (1990) is "uniquely suited as it is the only one that quantitates statistically significant interactions." As we hope we demonstrated in our review, their conclusion is overstated.

K. Nonparametric Response Surface Approaches

There are many response surface approaches available that do not require an a priori assumption of a specific functional form containing estimable parameters. The method of Prichard and Shipman (1990) is a particularly simple nonparametric technique, which connects data points with straight lines. More sophisticated nonparametric approaches that have been applied to concentration-effect data include: kernel estimation (Staniswalis, 1989), spline-based procedures for monotone curve smoothing (Kelly and Rice, 1990), and a more traditional spline-based procedure introduced by Sühnel (1990) and later applied by Baumgart et al. (1991).

Laska et al. (1994) published an approach to detect Loewe synergism or Loewe antgonism, which uses some geometrical principles derived from Loewe additivity response surfaces, but which does not require assumptions regarding the specific functional form of the individual dose-response curves or the combined-action surface. Thus, the approach uses a nonparametric structural model. The random model used to describe data variation can be either parametric or nonparametric. A minimum of only three design points are needed to apply this method; it should be classified as an hypothesis-testing rather a response surface approach.

Only the traditional spline-based response surface approach will be reviewed here.

1. Bivariate spline fitting (Sühnel, 1990). Essentially, Sühnel (1990) proposed to fit data from combination experiments with bivariate splines, without and with smoothing, and then to display the resulting 3-D surface and contours at various levels of the surface. Bivariate splines are sets of piecewise polynomials running in two dimensions that flexibly follow the points of a surface. The raw data from the common data set is shown in figure 21(A) with a bivariate spline (Harder and Desmaris, 1972; Meinguet, 1979), with no smoothing, fit to the data with the procedure, G3GRID from the SAS statistical package (SAS Institute, 1987). Figure 21(B) shows contours drawn from the raw data at 10% effect intervals (from 90% to 0% Control, from left to right), using the SAS procedure, GCONTOUR, using an algorithm from Snyder (1978). Sühnel emphasizes that the shape of the contours can be interpreted directly without the need of fitting a parametric function to the data. A straight diagonal NW-SE isobol would be consistent with Loewe additivity. Because the isobols in figure 21(B) are mostly slightly bowed downward, the conclusion is slight Loewe synergism. The approach is a more sophisticated version of the Prichard and Shipman (1990) approach, but with the null reference model being Loewe additivity, not Bliss independence. The Sühnel (1990) approach shares many of the advantages and disadvantages of the Prichard and Shipman (1990) approach.

The main advantages include:

(*a*) Loewe additivity is the null reference model.

(*b*) the approach is very flexible and does not require a parametric model for either the single agent concentration-effect curves, or for combined-action. Mosaics of interspersed regions of varying degrees of both Loewe synergism and Bliss antagonism are easily accommodated. Sühnel (1992a, 1992b) considers this characteristic so important that he has questioned the routine use of 3-D combined-action models, such as Eq. 5, which include only a single interaction parameter.

The disadvantages include:

(*a*) like many nonparametric response surface approaches, the required experimental design must include a large number of regularly dispersed points.

(*b*) the approach is essentially only an exploratory approach.

(*c*) no summary measures of interaction intensity or conclusion uncertainty are provided.

(*d*) the approach is more complex to implement and to use than the Prichard and Shipman (1990) approach.

(*e*) the potential user is required to find his own software implementation of the approach.

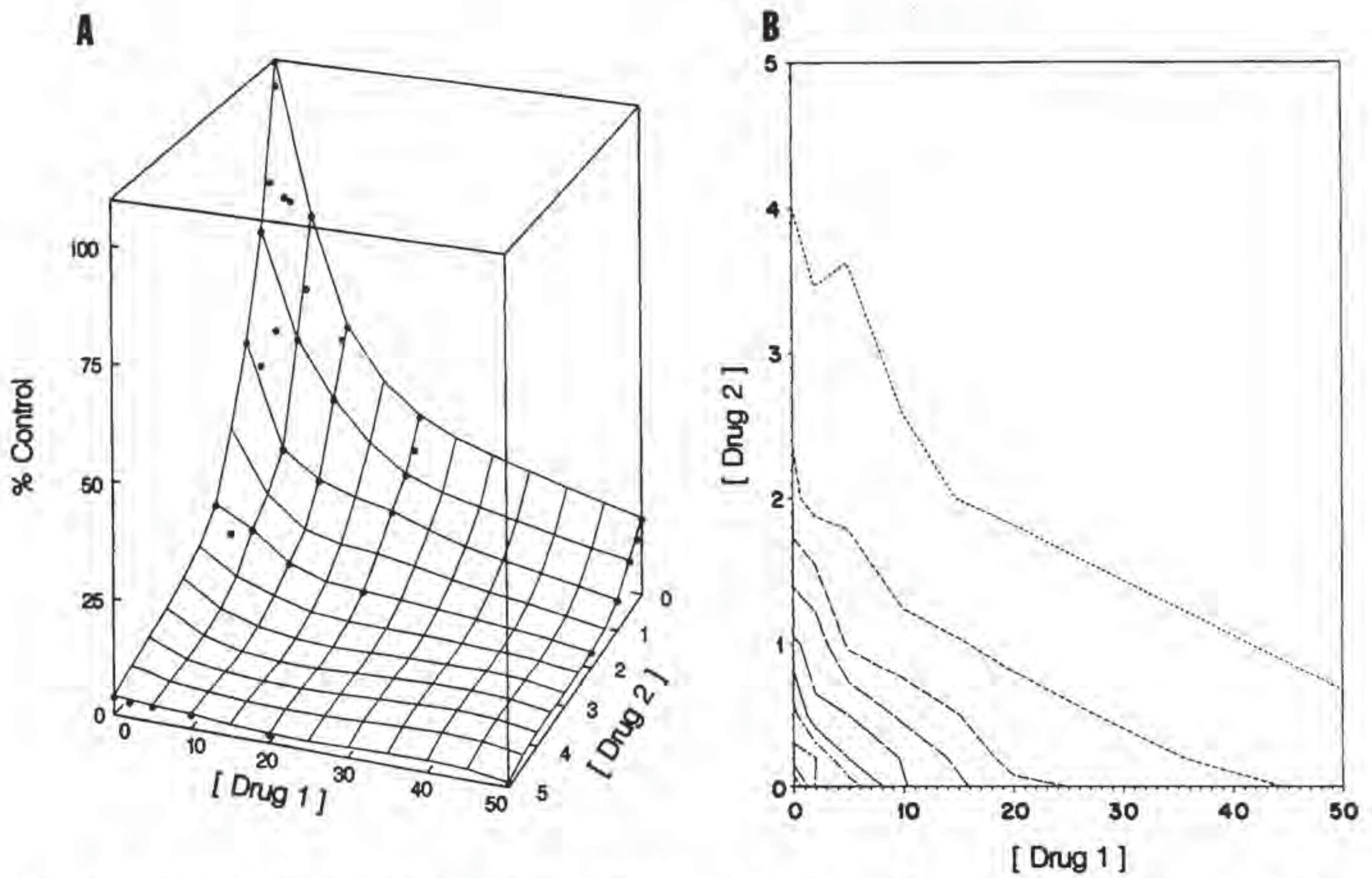


FIG. 21. Analysis of data from table 3, columns 2 through 4 by a nonparametric approach interpreted from Sühnel (1990). (*A*) The surface is a fit of the data with a bivariate spline (Harder and Desmarais, 1972; Meinguet, 1979), no smoothing, with the procedure, G3GRID, from SAS (SAS Institute, 1987). All 38 data points, whether they fall above or below the surface, are shown as solid circles. (*B*) Contours drawn from the raw data at 10% effect intervals (from 90% to 0%Control, from left to right), using the SAS procedure, GCONTOUR, using an algorithm from Snyder (1978). The general shape of the contours is in the direction of Loewe synergism.

L. Parametric Response Surface Approaches

In many senses, parametric response surface approaches are the most complex and difficult to apply to the problem of the joint action of agents. They may require the scientist-user to be facile with terminology and concepts that were not part of his formal education, may require the consultative advice of a statistician or other quantitative professional, and will require computing facilities and expertise. However, in a broader sense, these approaches may be the simplest of all of the methods discussed so far. In general, to apply the approaches, (*a*) logical models are fit to data with automated computer programs, (*b*) parameter estimates, other statistics, and graphs (3-D and 2-D) are generated and interpreted, (*c*) conclusions are made.

1. Models of Greco et al. (1990). Eq. 5 and close variants have been successfully applied to laboratory data from several studies (e.g., Greco et al., 1990; Gaumont et al., 1992; Greco and Dembinski, 1992; Greco and Rustum, 1992; Guimarães et al., 1994). Eq. 5 was fit to the common data set with nonlinear regression, weighted by the reciprocal of the square of the predicted effect. [Metzler (1981) provides a good description of nonlinear regression intended for biomedical scientists.] The Nash (1979) version of the Marquardt (1963) algorithm for nonlinear regression was coded by our group in MicroSoft FORTRAN, and run on MSDOS-compatible microcomputers.

The six best-fit parameter estimates (± standard error) were: $Econ$ = 95.1 ± 4.5; $IC_{50,1}$ = 11.1 ± 1.3; $IC_{50,2}$ = 1.07 ± 0.068; m_1 = -1.05 ± 0.078; m_2 = -2.04 ± 0.080; α = 0.519 ± 0.11. The 95% confidence intervals for each parameter can be calculated by multiplying each standard error by the appropriate value of the Student's t-test distribution and then adding and subtracting this value from the parameter estimate. The appropriate value of the $t_{0.025}$ distribution for two-sided 95% confidence intervals and 32 degrees of freedom (38 data points, 6 parameters) is 2.04. The 95% confidence intervals were: $Econ$, 86.0 to 104; $IC_{50,1}$, 8.40 to 13.9; $IC_{50,2}$, 0.934 to 1.21; m_1, -1.21 to -0.892; m_2, -2.20 to -1.88; α, 0.300 to 0.738. None of the 95% confidence intervals encompass zero; all of the parameters were well estimated. This is a positive indication of the model fitting the data well.

The raw data and best fit 3-D curve are shown in figure 22(A). A 2-D representation of the same concentration-effect surface is shown in the isobologram of figure 23, which was formed by the intersection of the surface with planes at 10, 50, 90, and 99% inhibition. Figure 24 includes concentration-effect curves (logarithmic concentration scales) for drug 1 at different drug 2 concentrations (left panel) and for drug 2 at different drug 1 concentrations (right panel). The curves are simulations of Eq. 5 with the best-fit estimated parameters. The curves are intersections of the surface shown in figure 22(A) with vertical planes at the concentrations of drug 2 and drug 1 listed in the figure. These curves, along with the actual data points, provide a visual analysis of the goodness of fit. Note the differences between the set of best-fit simulated curves in figure 24 and the analogous hand-drawn curves in figure 13. Figure 25 shows concentration-effect curves simulated with the best-fit parameters for drug 1, drug 2, a 10:1 mixture of drug 1 to drug 2, and a 10:1 mixture with the assumption of Loewe additivity (α = 0). This 2-D representation of the full 3-D surface in figure 22(A) provides a visual assessment of the magnitude of the shift of the concentration-effect curves, because of Loewe synergism, for fixed ratio mixtures. The IC_{50} value for the 10:1 mixture of the Loewe synergistic combination was 1.015-fold (5.45/5.37) lower than the expected value for the Loewe additive combination. This is close to the ratio of 1.012-fold (5.00/4.94) for ideal data containing no error. It is apparent that an α value of 0.5 leads to only subtle shifts in mixture concentration-effect curves. Because the α estimate is positive and the 95% confidence interval,

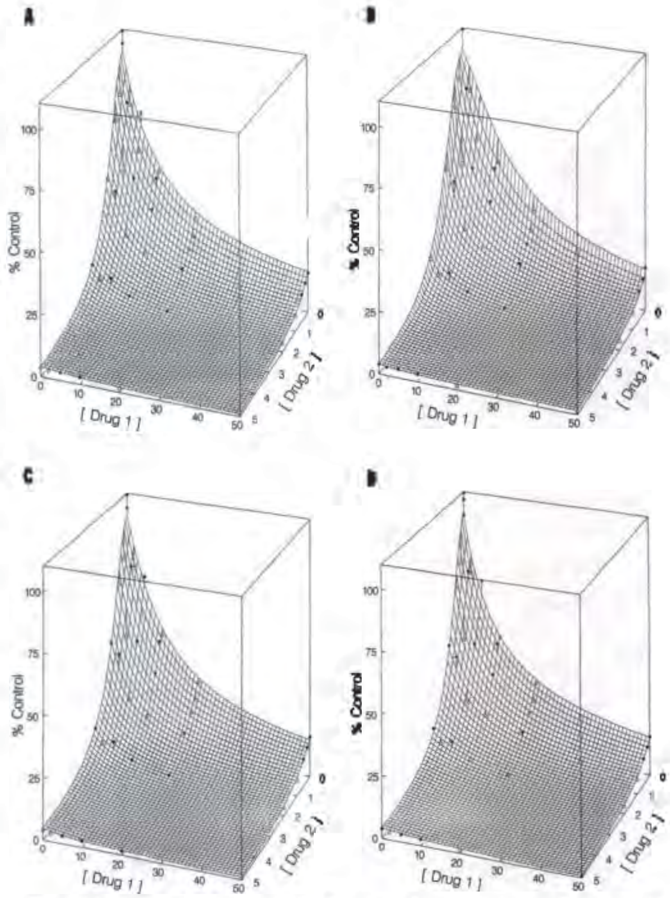


FIG. 22. 3-D concentration-effect surfaces estimated from the best fit of four different models, with weighted nonlinear regression as described in the text, to the data from table 3, columns 2 through 4. Both fitted and raw data are expressed as a percentage of the estimated Econ parameter. Solid points are above the surface; open points fall below the surface. (*A*) Eq. 5; (*B*) Eq. 28; (*C*) Eq. 29; (*D*) Eq. 29.

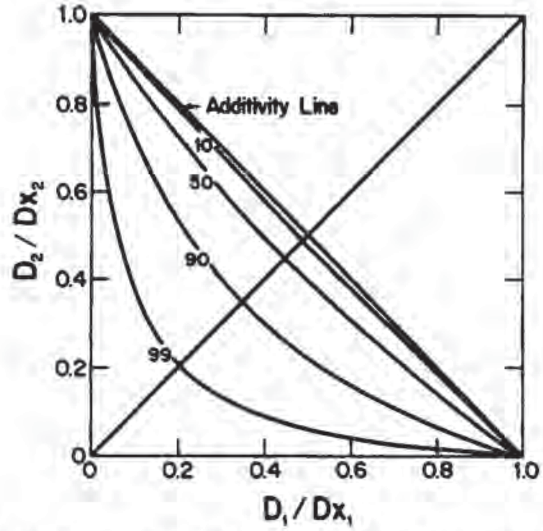


FIG. 23. Families of 2-D isobols for the best fit of Eq. 5 to the data from table 3, columns 2 through 4. The set of contours is a 2-D representation of the 3-D response surface in figure 22, panel (A). Note that the X- and Y-axes are the concentrations of each drug transformed by division by the appropriate value of the dose (or concentration) of drug that inhibits survival by X% (D_X). The numbers on the isobols indicate the % inhibitory level.

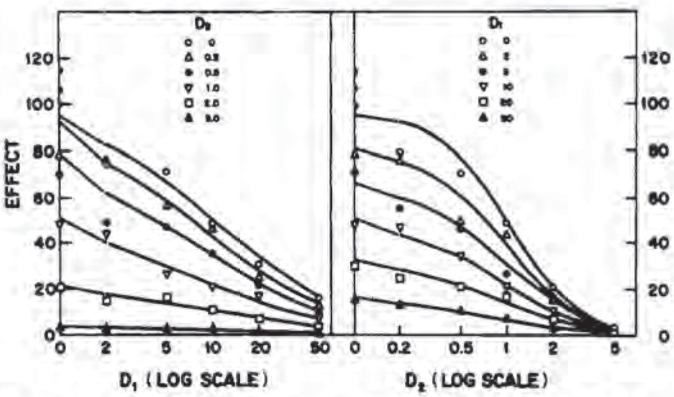


FIG. 24. Families of 2-D concentration-effect curves for the best fit of Eq. 5 to the data from table 3, columns 2 through 4. This is another 2-D representation of the 3-D response surface in figure 22, panel (A). Note that drug concentrations are on logarithmic scales.

0.300 to 0.738, does not encompass zero, a claim of small but significant synergism is made.

As was stated previously several times in this paper, Eq. 5, our flagship model, is *a* model for combined-action, not *the* model. Eq. 5 has a questionable property: for negative values of the interaction parameter, α, the 3-D concentration-effect surface has a saddle point and rises back to *Econ* at simultaneous high concentrations of both agents. This is illustrated in figure 26, a simulation of Eq. 5 with α = -1 (Loewe antagonism). Like the fit of second order polynomial models to data sets that show slight curvature, the fit of Eq. 5 to experimental data demonstrating Loewe antagonism may be valid for only a restricted region. The fit of Eq. 5 with negative α

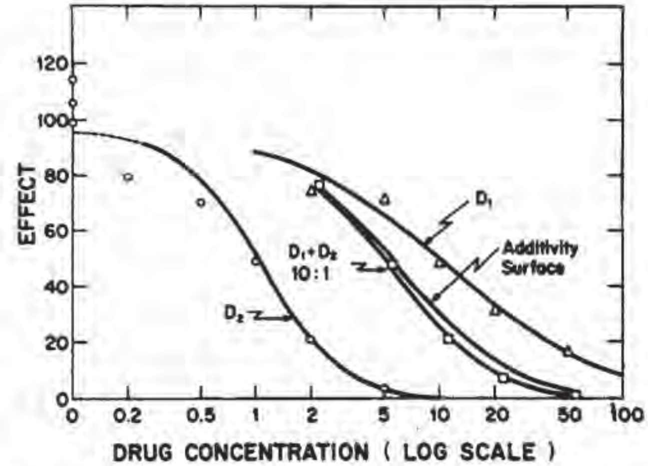


FIG. 25. Predicted 2-D concentration-effect curves for drug 1 alone, drug 2 alone, and the combination of drug 1 and 2 in a fixed 10:1 ratio for the best fit of Eq. 5 to the full data set from table 3, columns 2 through 4. The predicted Loewe additivity curve for the same combination at a fixed ratio of 10:1, simulated by setting α = 0, is also shown. The X-axis is the sum of concentrations of drug 1 and drug 2 (logarithmic scale). The raw data points are the same ones shown in figure 16.

estimates to experimental data has been shown to be satisfactory (e.g., Greco and Dembinski, 1992). However, we have systematically searched for a logical model that would not rise up at mixtures of high agent concentrations.

Such an experimental model is Eq. 28, whose general form was first suggested by Finney (1952) and later included in a list of plausible interaction models by Hewlett (1969). (Eq. 28 rises back toward *Econ* only at very high agent concentrations and large negative α values.) Eq. 28 is a specific example of the general Loewe combined-action model, Eq. 9. Eq. 28 differs from Eq. 5 by having all of the right-hand expression, except for α, raised to the ½ power. For simulations of Eq. 28, the extent of bowing will be the same for isobols at different effect levels determined from plots of $D_2/ID_{X,2}$ vs. $D_1/ID_{X,1}$. This is in contrast to the greater bowing of isobols at higher levels of inhibition for Eq. 5, as seen in figures 4(E), 5(A), 8(C), and 23.

$$1 = \frac{D_1}{IC_{50,1}\left(\dfrac{E}{Econ - E}\right)^{1/m_1}} + \frac{D_2}{IC_{50,2}\left(\dfrac{E}{Econ - E}\right)^{1/m_2}} + \alpha\left(\frac{D_1 D_2}{IC_{50,1} IC_{50,2}\left(\dfrac{E}{Econ - E}\right)^{(1/m_1 + 1/m_2)}}\right)^{1/2} \qquad [28]$$

Eq. 28 was fit to the common data set in the same way as described for Eq. 5. Figure 22(B) shows the best-fit 3-D surface and the raw data points. The six estimated parameters were: $Econ$ = 88.9 ± 5.5; $IC_{50,1}$= 15.6 ± 2.2;

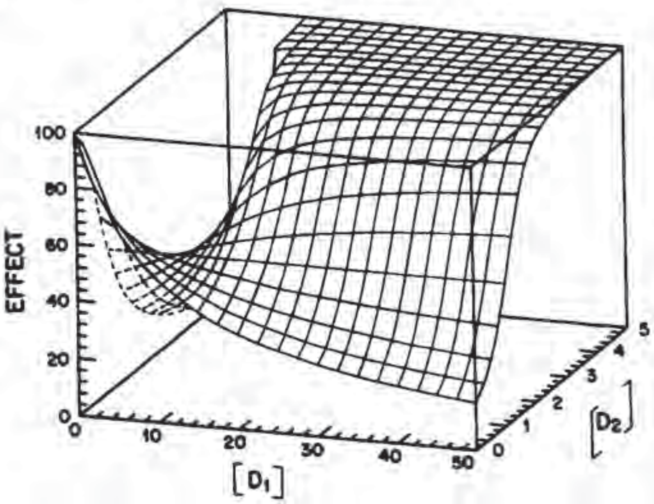


FIG. 26. Simulation of Eq. 5, with $Econ = 100$, $IC_{50,1} = 10$, $IC_{50,2} = 1$, $m_1 = -1$, $m_2 = -2$, $\alpha = -1$, an example of Loewe antagonism.

$IC_{50,2} = 1.27 \pm 0.11$; $m_1 = -1.34 \pm 0.11$; $m_2 = -2.28 \pm 0.13$; $\alpha = 0.643 \pm 0.18$. As seen in Figure 22(B) and in other 2-D plots not shown, the goodness of fit was adequate. Because α was positive and its 95% confidence interval did not encompass zero (0.270 to 1.02), Loewe synergism is claimed. [Note however, that for some negative values of α (from -1.414 to 0), the isobols simulated with Eq. 28 lie outside the limits of the graph of $D_2/ID_{X,2}$ vs. $D_1/ID_{X,1}$ shown in figure 5(A); i.e., they lie outside the unit square. This inadequacy of the general form of this model was first pointed out by Machado and Robinson (1994) and further explored by Khinkis and Greco (1994).]

2. Models of Weinstein et al. (1990). Eq. 29 was introduced by Weinstein et al. (1990) and Bunow and Weinstein (1990); a reparameterization has been used more recently (Kageyama et al. (1992). Eq. 29 is called the robust potentiation model. Loewe additivity is its null reference model. PC_1, PC_2, are the concentrations of agents 1, 2 required to increase the apparent potency of the other drug by a factor of 2. The parameters, bp_1, bp_2, govern the slope of the potentiative effect of agents 1 and 2, respectively. Loewe synergism, but not Loewe antagonism, can be modeled with Eq. 29, because a negative *PC* parameter cannot be used with a corresponding nonintegral *bp* parameter. Eq. 29 and several other models are integrated into the software package COMBO, which runs in the MLAB (Civilized Software Inc, 1991) environment on MSDOS-compatible microcomputers. We fit Eq. 29 to data with nonlinear regression with our FORTRAN program as described for Eqs. 5 and 28, with weights equal to the reciprocal of the square of the predicted response; we did not implement the interesting weighting scheme described by Bunow and Weinstein (1990), a Gaussian kernel windowing technique based on estimated responses.

We were unsuccessful in fitting the full nine-parameter model, Eq. 29 to the common data set. There was not enough information in the data set to allow the estimation of four separate interaction parameters, PC_1, PC_2, bp_1, and bp_2. However we were successful in fitting two different reduced seven-parameter models to the common data set. Figure 22(C) shows the fit of Eq. 29, with the expression containing PC_1 and bp_1 eliminated, and figure 22(D) shows the fit of Eq. 29, with the expression containing PC_2 and bp_2 eliminated. The need to use only one of the two pairs of interaction parameters was also reported by Weinstein et al. (1990). The estimated parameters for the best fit shown in figure 22(C) were: $Econ = 95.3 \pm 4.8$; $IC_{50,1} = 11.0 \pm 1.4$; $IC_{50,2} = 1.02 \pm 0.084$; $m_1 = -1.13 \pm 0.077$; $m_2 = -1.94 \pm 0.10$; $PC_2 = 1.65 \pm 0.31$; $bp_2 = 1.55 \pm 0.21$. The estimated parameters for the best fit shown in figure 22(D) were: $Econ = 98.9 \pm 4.5$; $IC_{50,1} = 9.49 \pm 1.2$; $IC_{50,2} = 0.947 \pm 0.059$; $m_1 = -1.00 \pm 0.064$; $m_2 = -1.92 \pm 0.066$; $PC_1 = 44.8 \pm 5.0$; $bp_1 = 1.18 \pm 0.16$. Because the fit was good for both reduced models, the interaction parameters, PC_1, PC_2, were both positive, and their 95% confidence intervals did not encompass zero, the conclusion is Loewe synergism.

$$1 = \frac{D_1\left(1 + \left(\frac{D_2}{PC_2}\right)^{bp_2}\right)}{IC_{50,1}\left(\frac{E}{Econ - E}\right)^{1/m_1}} + \frac{D_2\left(1 + \left(\frac{D_1}{PC_1}\right)^{bp_1}\right)}{IC_{50,2}\left(\frac{E}{Econ - E}\right)^{1/m_2}} \qquad [29]$$

There are many other parametric response surface models that could be applied to the common data set. Hewlett (1969) provides a general framework for deriving many specific, potentially useful, multivariate concentration-effect combined-action models. More recently, Machado and Robinson (1994) have reviewed this set of combined-action models, plus the general forms of Eqs. 5, 29, and an original model, Eq. 30. Eq. 30 has a single interaction parameter, which is called η. Unfortunately, like Eq.28, Eq. 30 has the disadvantage of having isobols lie outside the unit square of the graph of $D_2/ID_{X,2}$ vs. $D_1/ID_{X,1}$ for values of η from $-\infty$ to -0.333 and from 1 to ∞ (Khinkis and Greco, 1994).

$$1 = (1+\eta)\left[\frac{D_1}{IC_{50,1}\left(\frac{E}{Econ - E}\right)^{1/m_1}} + \frac{D_2}{IC_{50,2}\left(\frac{E}{Econ - E}\right)^{1/m_2}}\right] - \eta\left[\frac{D_1}{IC_{50,1}\left(\frac{E}{Econ - E}\right)^{1/m_1}} - \frac{D_2}{IC_{50,2}\left(\frac{E}{Econ - E}\right)^{1/m_2}}\right]^2 \qquad [30]$$

The response surface approaches have the following advantages:

(*a*) they provide a quantitative measure of the intensity of interaction, along with a measure of its uncertainty.

(*b*) they reduce the full data set from an experiment to a smaller set of parameters, along with uncertainty estimates.

(*c*) they facilitate prediction of the response under new conditions.

(*d*) they are appropriate for complex situations, such as three-, four-, and five-drug combinations.

(*e*) they aid in experimental design, including the design of complex experiments. Also, they tend to be tolerant of a wide spectrum of designs.

(*f*) they have the potential to explain, in intimate detail, all of the characteristics of a complex system, and thereby facilitate a deep understanding of the system.

(*g*) they are objective (relatively), rigorous, and consistent with modern statistical theory. In addition to the brief statistical summary provided for the fits of Eq. 5, 28, and 29 to the common data set, there are other useful statistical diagnostics available, including overall goodness of fit statistics, confidence envelopes around the fitted surface, and residual (functions of the difference between the actual and fitted data) analyses (e.g., McCullagh and Nelder 1989; Seber and Wild, 1989; Bates and Watts, 1988; Carter et al., 1986; Machado and Robinson, 1994).

(*h*) parametric 3-D concentration-effect models may be used as the pharmacodynamic component of composite pharmacokinetic-pharmacodynamic models, to be used for the clinical study of the disposition and effect of drug combinations.

(*i*) finally, as described in Section III, response surface approaches are useful in explaining the similarities and differences among other rival approaches to the assessment of combined-action.

The four panels of figure 22 look very similar. From the statistics provided for the fit of Eqs. 5, 28 and 29 to the common data set, it would be difficult to choose the best structural model. To a great extent, the exact form of combined-action models is arbitrary, and considerations other than the goodness of fit of a model to a specific data set, must be used to decide upon a modeling framework. These criteria include:

(*a*) a model should allow the "slope" for each agent's individual concentration-effect curve to be different; this is allowed by Eqs. 5, 28, 29, 30.

(*b*) it is desirable to allow each agent's individual concentration-effect curve to have a different functional form; however, the need for such a model seldom arises.

(*c*) the model should be one from a hierarchical set, which allows expansion and reduction of models by inclusion and deletion of expressions and parameters, in a logical, hierarchical manner. For example, a model might be expanded to accommodate more than two agents, or to describe simultaneous Loewe synergism and Loewe antagonism in different regions of the concentration-effect surface, and reduced to describe an agent that increases the pharmacological effect of a second agent, but which has no effect by itself (synergism, see table 1).

(*d*) the simulation of the model should present no unsolvable numerical problems. For example, Eqs. 5, 28–30 all require appropriate one-dimensional root finders (e.g., Thisted, 1988), but these are easily programmed, and have been found to be reliable.

(*e*) if normalized isobols [e.g., fig. 8(C)] for typical data increase in bowing at higher levels of inhibition, then this characteristic should be intrinsic to the model.

(*f*) a model should have the fewest parameters possible to adequately describe combined-action data.

(*g*) it is desirable for the parameters to have some geometrical meaning; i.e., upon hearing of the values of a model's parameters, an experienced researcher should be able to mentally picture 2-D and 3-D concentration-effect curves. This would be true for Eqs. 5 and 28 through 30.

(*h*) it is desirable for the model to follow the correct course, even in regions for which there is no data. In other words, cautious extrapolation should be possible.

(*i*) the modeling paradigm should allow the combining of a 3-D concentration-effect structural model, such as Eqs. 5 and 28 through 30, with an appropriate random model, for fitting data with modern statistical approaches, such as maximum likelihood estimation.

(*j*) in general, the structural model should closely follow the overall average data, without following random fluctuations.

(*k*) the isobols for the model should lie within the unit square of the graph of $D_2/ID_{X,2}$ vs. $D_1/ID_{X,1}$ for all values of the interaction parameter(s). This last criteria is not met by Eqs. 28 and 30.

A critical area of future research will be the derivation, collection, and comparison of rival multivariate parametric concentration-effect combined-action models. A comprehensive critical comparison of rival models (e.g., Eqs. 5 and 28 through 30) is beyond the scope of this review. Machado and Robinson (1994) present one of the first such critical reviews; our group is also currently working in this area (Khinkis and Greco, 1994). Although the field of response surface modeling of agent interactions has old roots (e.g., Finney, 1952), only in recent years has the availability of computer hardware and software made it into a practical, universally important discipline.

The disadvantages of fitting 3-D parametric concentration-effect models to data include:

(*a*) there are an infinite number of plausible parametric models; it may be difficult to choose among rival models. Different rival models may lead to different conclusions. The parametric modeling paradigm is still evolving; an analysis of data with a current model might be proven to be suboptimal at a later time.

(*b*) the proper fitting of these models to data requires statistical and computing expertise and adequate computer hardware and software. However, the acquisition of these skills and tools is increasing among laboratory scientists, and, in our view, is very cost-effective. In addition, as an alternate solution, both initial and long-term collaborations between laboratory and quantitative scientists can be very Loewe synergistic.

(c) the links between empirical models of combined-action, such as Eqs. 5 and 28 through 30, and theoretical mechanistic models of molecular, biochemical, and physiological systems have not been systematically made. In other words, after one makes a rigorous claim of, let's say, Loewe synergism, it is in no way obvious what this implies regarding the mechanistic interaction of two agents. Some work has been done in this field (e.g., Werkheiser et al., 1973; Jackson, 1980, 1984, 1991, 1992, 1993; Bravo et al., 1992). However, this critical research area is in its infancy.

VI. Comparison of Rival Approaches for Discrete Success/Failure Data

This section will discuss approaches to the assessment of the combined-action of agents, in which the measured or observed response is binary (quantal); i.e., it is success or failure, yes or no, dead or alive, on or off, 0 or 1. The data is often grouped by treatment and is expressed as a proportion of successes; e.g., five successes of eight trials, or 0.625. Most of the material in this section is from Greco (1989). A random model that describes the statistical variation in success/failure data is the Bernoulli distribution, and one that describes the variation in proportion data is the binomial distribution (Larson, 1982). Figure 3 showed a concentration-effect structural curve with binomial variation about one point on the curve. A formula for the binomial model is Eq. 31, in which n is the number of attempts in a binomial trial, k is the number of successes, Y is the proportion of successes ($Y = k/n$), y is a particular value of Y ($y = 0, 1/n, 2/n, \ldots, 1$), μ is the mean or expected value of Y, $P(Y = y)$ is the probability that the general Y variable will equal the particular value y, and ( ) is the combination of n things taken ny at a time. [Note: Eq. 31 is different from but equivalent to the more common form of the binomial distribution equation (e.g., Larson, 1982) not shown here. We reparameterized the more common form into Eq. 31 to facilitate the combining of structural with random models.] Because the overall mean or expected value of Y is merely the value of the structural model, structural models for success/failure concentration-effect phenomena can be generated by simply substituting μ for E in any of the structural concentration-effect models previously described in this paper for continuous data. For example, the Hill model can be expressed as Eq. 32, and our flagship combined-action model can be expressed as Eq. 33. Note that the *Econ* parameter has been constrained to be the constant, 1, in Eqs. 32 and 33. In order to make a composite structural-random model for data fitting, the structural expression for μ is inserted into the binomial model, Eq. 31, either directly or indirectly with a numerical procedure.

$$P(Y = y) = \binom{n}{ny}\mu^{ny}(1-\mu)^{n(1-y)} \qquad [31]$$

$$\mu = \frac{\left[\dfrac{D}{Dm}\right]^m}{1 + \left[\dfrac{D}{Dm}\right]^m} \qquad [32]$$

$$1 = \frac{D_1}{Dm_1\left[\dfrac{\mu}{1-\mu}\right]^{1/m_1}} + \frac{D_2}{Dm_2\left[\dfrac{\mu}{1-\mu}\right]^{1/m_2}} + \frac{\alpha D_1 D_2}{Dm_1 Dm_2\left[\dfrac{\mu}{1-\mu}\right]^{(1/2m_1+1/2m_2)}} \qquad [33]$$

Rival approaches for the assessment of combined-action when the response is quantal (proportions of success/failure) will be compared in a manner similar to the comparison in Section V of rival approaches for combined-action when the response is a continuous measure. A simulated data set for a pair of inhibitory drugs, listed in table 4, was generated by first calculating μ with Eq. 33 with parameters, $IC_{50,1} = 10$, $IC_{50,2} = 1$, $m_1 = -1$, $m_2 = -2$, $\alpha = 1$; and then entering μ, along with n into a binomial random number generator from the Statgraphics Software Package (STSC Inc., 1988). This data set will be analyzed with three different approaches, the approach of Gessner (1974), the fitting of the parametric response surface model, Eq. 33 (Greco and Lawrence, 1988) to the full data set, and the fitting of the multivariate linear logistic model (Cox, 1970), Eq. 34, to the full data set (e.g., Carter et al., 1983, 1988; Brunden et al., 1988).

$$\mu = \frac{\exp(\beta_0 + \beta_1 D_1 + \beta_2 D_2 + \beta_{12} D_1 D_2)}{1 + \exp(\beta_0 + \beta_1 D_1 + \beta_2 D_2 + \beta_{12} D_1 D_2)} \qquad [34]$$

Many of the methods for analyzing continuous combined-action data, described in Section V, could be used, and have been previously used, for analyzing proportion data. If one merely calculates the proportions of survivors from table 4 as decimal numbers and then treats these numbers as continuous data, then methods E.1 through E.11 could be directly applied without any additional complications. However, the variation pattern (probability distribution) of proportion data is fundamentally different from that for typical continuous biological data. For proportion data, usually the numbers of survivors and the total numbers of organisms undergoing a treatment is known without error. The variation in responses is usually caused by the fundamental nature of discrete binary responses; the variation is usually wider in the ID_{50} range of the concentration-effect curve

TABLE 4
Data set, with a binary (proportion) response variable, used for comparison of rival data analysis approaches

D_1	D_2	Number of survivors*	Total number of organisms
0.1	0	100	100
0.3	0	97	100
0.5	0	96	100
1	0	96	100
3	0	72	100
5	0	59	100
10	0	57	100
30	0	32	100
50	0	13	100
100	0	13	100
0	0.01	100	100
0	0.03	100	100
0	0.05	99	100
0	0.1	98	100
0	0.3	95	100
0	0.5	72	100
0	1	46	100
0	3	6	100
0	5	3	100
0	10	2	100
0.1	0.01	99	100
0.3	0.03	97	100
0.5	0.05	94	100
1	0.1	87	100
3	0.3	59	100
5	0.5	53	100
10	1	24	100
30	3	7	100
50	5	2	100
100	10	0	100

* The number of survivors in column 3 was generated by (*a*) calculating μ with Eq. 33 with parameters, $IC_{50,1} = 10$, $IC_{50,2} = 1$, $m_1 = -1$, $m_2 = -2$, $\alpha = 1$; then entering μ, along with n (the total number of organisms, equal to 100) into a binomial random number generator from the Statgraphics Software Package (STSC Inc., 1986).

and smaller near the two ends of the curve. Proportions above 1 and below 0 do not exist. In contrast, continuous biological data often follow bell-shaped normal distributions, with larger variances associated with larger measurements (proportional error, constant coefficient of variation). Individual measurements (% control) both above 100% and below 0% often occur. Proportions will tend to become normally distributed as n becomes large, and as the true proportion tends away from the ends of the range, 0 and 1. Methods, E.1 through E.11, which ignore the true random component of the data, will only be, at best, approximately correct for binary data. However, they can provide very useful preliminary exploratory procedures. Nonetheless, only approaches that fully exploit the binary nature of the data will be compared in this section.

Much of the early work on the problem of combined-action of agents was focused on biological systems with quantal responses (e.g., Bliss, 1939; Finney, 1952, 1971; Hewlett and Plackett, 1959, 1979; Hewlett, 1969; Plackett and Hewlett, 1948, 1952, 1967). (It seems that systems with quantal responses were of more interest to statisticians, whereas systems with continuous responses have been of more interest to pharmacologists.) Specific approaches and models of these pioneers in the field of combined-action assessment will not be reviewed in this paper. However, many of their concepts, approaches and models form the basis of the three approaches that will be compared.

A. Approach of Gessner (1974)

Our interpretation of the method of Gessner (1974) first consists of fitting appropriate single agent models to the data for agent 1 alone, agent 2 alone, and fixed ratios of D_1:D_2. Gessner (1974) recommends the probit model (e.g., Finney, 1952), Eq. 35, for this purpose; however, we also explored the use of the univariate linear logistic model with ln(D) as the input, Eq. 36, and the univariate linear logistic model with D as the input, Eq. 37. Note that Eq. 32 and 36 are different parameterizations of the same fundamental model, in which β_0 = $-m\ln(Dm)$ and $\beta_1 = m$. These three models were fit to the data for drug 1 alone, drug 2 alone, and the 10:1 mixture from table 4, with maximum likelihood estimation via nonlinear least squares (Jennrich and Moore, 1975), with the software package, PCNONLIN (Statistical Consultants, Inc., 1986), on an MSDOS-compatible microcomputer. The best fit of Eq. 32, and the equivalent model, Eq. 36, to the three sets of data points from the common 30-point data set, is shown in figure 27(A). The best fits of Eqs. 35 and 37 are shown in figures 27(B) and 27(C), respectively. The fits look good for Eqs. 32, 36, and 35, but not for Eq. 37. The parameter estimates ± standard errors for the fits of these four models were: (for Eq. 32, drug 1, Dm = 10.7 ± 0.99, m = -0.982 ± 0.060; drug 2, Dm = 0.895 ± 0.056, m = -1.99 ± 0.14; drug 1+2, Dm = 4.36 ± 0.30, m = -1.59 ± 0.10). (For Eq. 36, drug 1, β_0 = 2.33 ± 0.16, β_1 = -0.982 ± 0.060; drug 2, β_0 = -0.220 ± 0.13, β_1 = -1.99 ± 0.14; drug 1+2, β_0 = 2.34 ± 0.19, β_1 = -1.59 ± 0.10). (For Eq. 35, drug 1, β_0 = 6.36 ± 0.084, β_1 = -1.32 ± 0.072; drug 2, β_0 = 4.90 ± 0.072, β_1 = -2.72 ± 0.17; drug 1+2, β_0 = 6.35 ± 0.10, β_1 = -2.12 ± 0.12). (For Eq. 37, drug 1, β_0 = 1.61 ± 0.10, β_1 = -0.0577 ± 0.0043; drug 2, β_0 = 2.55 ± 0.14, β_1 = -1.67 ± 0.13; drug 1+2, β_0 = 2.52 ± 0.16, β_1 = -0.403 ± 0.032). None of the 95% confidence intervals for any of the parameters for any of the models encompassed zero.

$$\text{Probit}(\mu) = \beta_0 + \beta_1 \log(D) \qquad [35]$$

$$\mu = \frac{\exp(\beta_0 + \beta_1 \ln(D))}{1 + \exp(\beta_0 + \beta_1 \ln(D))} \qquad [36]$$

$$\mu = \frac{\exp(\beta_0 + \beta_1 D)}{1 + \exp(\beta_0 + \beta_1 D)} \qquad [37]$$

The second stage of the method of Gessner (1974) is to plot the estimated Dm (ID_{50}) values, along with their

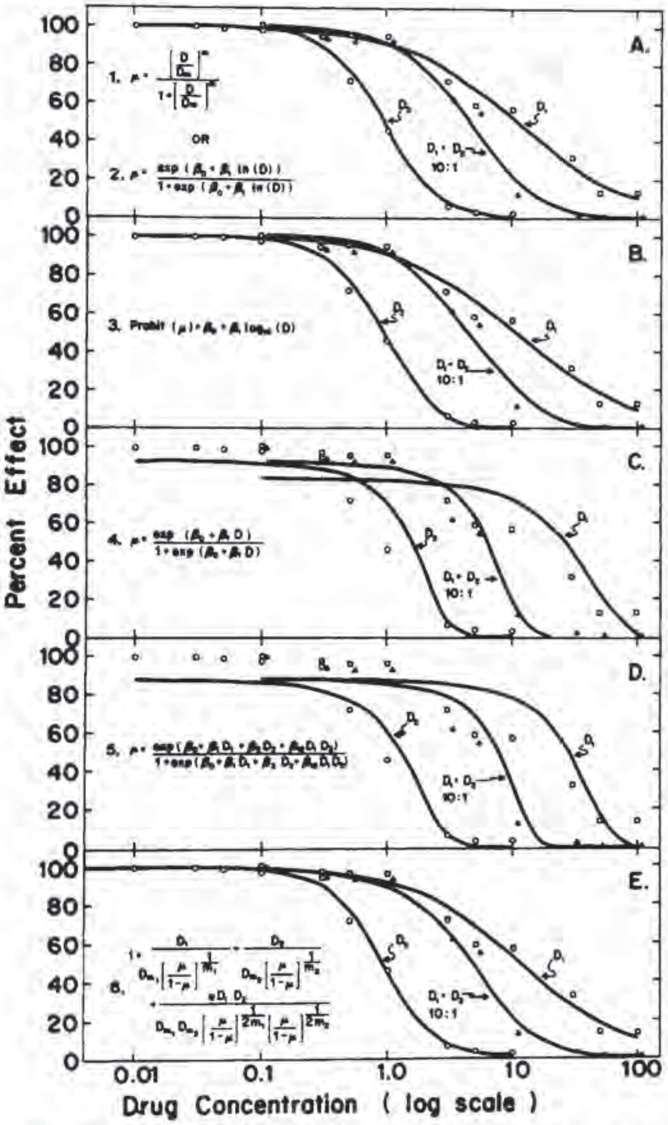


FIG. 27. Fitted curves of various models to the simulated data from table 4. See details in the text.

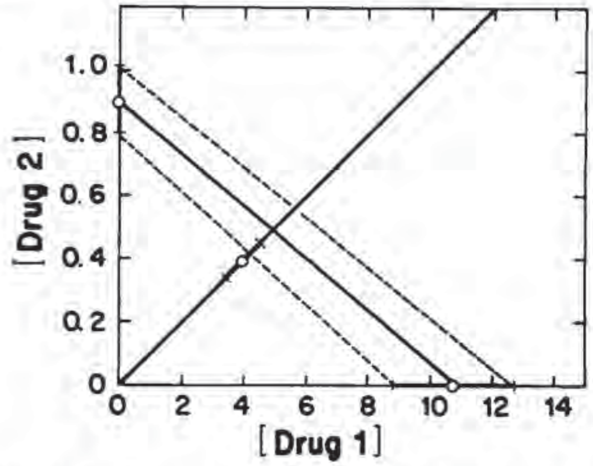


FIG. 28. Further isobolographic analysis of data from figure 27, panels (*A*), (*B*), using an interpretation of the approach of Gessner (1974).

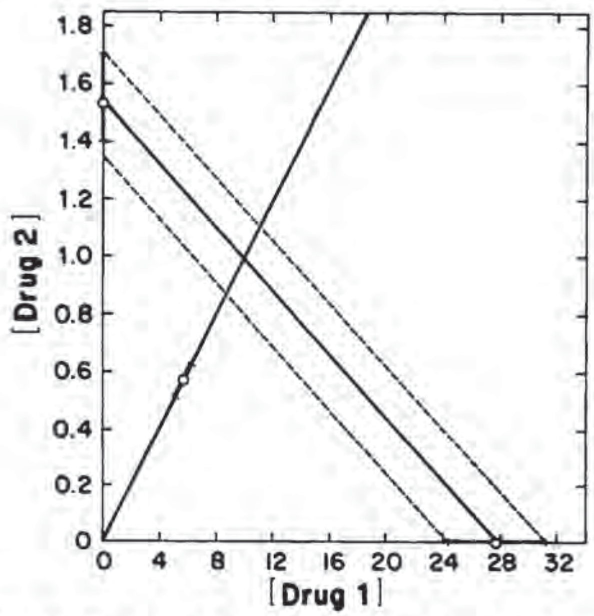


FIG. 29. Further isobolographic analysis of data from figure 27, panel (*C*), using an interpretation of the approach of Gessner (1974).

95% confidence intervals, for drug 1 alone, drug 2 alone, and for the mixture, on isobolograms. Figure 28 shows the isobolograms for the fits of Eqs. 32, 35 and 36, which all coincide, and figure 29 shows the isobologram for the fit of Eq. 37. The dashed lines connecting the ends of the 95% confidence intervals for the ID_{50}'s of drug 1 alone and drug 2 alone define a Loewe additivity region. Because the 95% confidence interval for the 10:1 mixture of drug 1+2 intersects the Loewe additivity region, a conclusion of Loewe additivity is made. In contrast, the isobologram replot for the fit of Eq. 37, figure 29, indicates Loewe synergism. Interestingly, the poor fit of Eq. 37 to the data resulted in poor estimates of the ID_{50}'s for each drug alone, and this lead to the "correct" claim of Loewe synergism. The overall conclusion for the method of Gessner (1974), based upon the fits of Eqs. 32, 35 or 36, and the isobologram replot in figure 28, is Loewe additivity.

It is also clear that the linear logistic model without the logarithmic transformation of the dose, Eq. 37, does not seem to have the ideal shape for typical concentration-effect data. It seems to miss points near 100% survival, and misses points for concentration-effect curves with relatively shallow slopes (around m = -1).

The advantages of the method of Gessner (1974) include:

(*a*) the underlying null reference model is Loewe additivity.

(*b*) the approach takes into account in an appropriate manner, the binomial variation of proportion data.

(*c*) the approach allows the slopes of the individual concentration-effect curves to be different.

(*d*) the derivation and application of complex full combined-action models are not necessary.

(*e* the isobologram replot is visual and intuitive.

(*f*) uncertainty measures, the 95% confidence intervals about the ID_{50}s, are included in the analysis.

(*g*) the approach can accommodate interspersed regions of Loewe synergism and antagonism.

(*h*) the general concepts of estimating ID_{50}s, along with 95% confidence intervals, and making a replot isobologram, are very general, and could be applied to continuous data.

(*i*) the approach is relatively easy to implement with standard software.

(*j*) the approach is an excellent front-end for more advanced model-fitting approaches and may provide the best final analysis for complex situations in which the degree of Loewe synergism and Loewe antagonism varies across the 3-D concentration-effect surface.

The disadvantages include:

(*a*) the additivity region bounded by the dashed lines connecting the ends of the 95% confidence intervals of the individual agent ID_{50}s was not created with a rigorous statistical derivation. The additivity region will tend to be too wide, too conservative, resulting in rejection of true Loewe synergism and Loewe antagonism too often. More realistic confidence bounds, based upon modern statistical theory, have been derived by Carter's group (Carter et al., 1986, 1988; Gennings et al., 1990).

(*b*) it is likely that the fitting of data for a fixed ratio of D_1:D_2 by concentration-effect models appropriate for single agents, such as Eqs. 32, 35, or 36, will result in biases, similar to the problems described for fitting the median-effect model to fixed ratio data, described in Appendix B, and in figure 17. We predict that the misfits will become more severe as the difference in slope parameters increases and as the intensity of interaction increases, as shown for the median-effect model, in figure 17. However, we predict, as indicated for the median-effect model in Appendix B, that the problems will tend to be minor if one focuses mainly on the ID_{50}s.

(*c*) maximum use is not made of the data, as compared with approaches centered on the fitting of full combined-action concentration-effect surfaces to all of the data simultaneously.

(*d*) summary measures of the intensity of interaction, along with uncertainty measures, are not provided.

An additional criticism—with which we take issue—leveled at the method of Gessner (1974) is that the approach does not adjust the 95% Loewe additivity region to take into account the problem of making multiple comparisons of ID_{50}s from several separate fixed ratio concentration-effect curves (Carter et al., 1988). Carter's group argues: "The procedure described suffers from the same problem associated with making multiple [Student's] t-tests to compare the means of a number of treatment groups. In such cases, the probability of incorrectly rejecting the null hypothesis of equality of treatment means is inflated. Here, the null hypothesis is one of additivity. Hence, the probability of incorrectly rejecting additivity and thereby concluding synergism is inflated."

We respond to this criticism by pointing out that, when applying Gessner's (1974) approach to datasets with several fixed ratios of D_1:D_2, the pattern of ID_{50} confidence intervals is taken into account; albeit, in an ad hoc manner, when making a conclusion. Each ID_{50} confidence interval is not meant to be interpreted in isolation. For example, if there were 10 different fixed ratios for our common data set, and if their ID_{50} confidence intervals were plotted in figure 29, and if a random assortment of significant Loewe synergism and Loewe antagonism were demonstrated, one would conclude either that the combined-action was very complex or that some errors were made in conducting the experiment. The experiment would probably be repeated. If only 1 of 10 fixed ratios showed significant Loewe synergism, with no apparent trend in the ID_{50} estimates, then the Loewe synergism would be considered suggestive at best, possibly a random artifact, and, if possible, the experiment would be repeated with larger sample sizes, especially in the region of suspected Loewe synergism. However, with the more probable result of consistent patterns of Loewe synergism or Loewe antagonism, (e.g., Gessner, 1988), the clusters of Loewe synergistic and/or Loewe antagonistic ID_{50} intervals will reinforce each other, leading to a more conservative, not to a more liberal, conclusion. The use of improperly inflated P-values, and conversely, improperly deflated 95% confidence intervals, caused by the making of multiple statistical comparisons, is certainly an important general problem in biostatistics (Miller, 1981). However, the problem is not relevant to the application of Gessner's approach when rationally applied to agent combination data.

B. Parametric Response Surface Approaches

Just as for continuous data, full 3-D combined-action concentration-effect models can be fit to proportion data, to assess the nature and intensity of agent interaction. The use of two different structural models will be demonstrated: our flagship combined-action model, Eq. 33, and the multivariate linear logistic model, Eq. 34. In principle, the general form of Eq. 28, Eq. 29 (Weinstein et al., 1990), Eq. 30 (Machado and Robinson, 1994), and any of the models reviewed by Hewlett (1969) could also be tried, but these are not included in this part of the review.

1. Model of Greco and Lawrence (1988). Eq. 33 was fit to the full common data set in table 4 with maximum likelihood estimation in the same manner as described in Section VI.A. The best fit surface is shown as three curves in figure 27(E). The fitted surface hugs the raw data, with a random distribution of points about the surface. The parameter estimates ± standard errors were: $Dm_1 = 11.2 \pm 0.99$, $m_1 = -0.995 \pm 0.052$, $Dm_2 = 0.905 \pm 0.056$, $m_2 = -2.05 \pm 0.14$, $\alpha = 0.903 \pm 0.46$. The 95% confidence interval for α was from 0.001 to 1.80. Therefore, Loewe synergism is claimed.

2. *Multivariate linear logistic model.* The use of the multivariate linear logistic model (Cox, 1970) is very popular in the analysis of clinical trial data and in Epidemiology, in cases in which the response variable is binary (Hosmer and Lemeshow, 1989). It is the most popular response surface model that has been routinely applied to quantal combined-action data (e.g., Carter et al., 1983, 1988; Brunden et al., 1988). Eq. 34, the multivariate linear logistic model for two agents, includes one interaction parameter, β_{12}. When β_{12} is positive, Loewe synergism is indicated; when β_{12} is negative, Loewe antagonism is indicated, and when β_{12} is zero, Loewe additivity is indicated. Eq. 34 was fit to the common data set in table 4 with the maximum likelihood approach described in Section VI.A. The fitted surface is shown in figure 27(D). The parameter estimates were: $\beta_0 = 2.03 \pm 0.071$, $\beta_1 = -0.0713 \pm 0.0043$, $\beta_2 = -1.54 \pm 0.11$, $\beta_{12} = -0.0837 \pm 0.025$. Because the 95% confidence interval for β_{12} is from -0.133 to -0.0347, Loewe antagonism might be concluded. However, the best fit of Eq. 34 to the data, shown in figure 27(D), does not look very good. First, the surface misses the points near 100% survival. Second, because the linear logistic model constrains agent 1 alone, agent 2 alone, and the 10:1 mixture all to have the same dose-effect slope (on a logarithmic dose scale), the data points are not randomly scattered about the curves; the surface systematically misses most of the data. These two characteristics make the multivariate linear logistic model suboptimal for assessing the combined-action of agents in many systems.

This second problem with the use of the linear logistic model, the constraining of the slopes of the concentration-effect curves, may have profound implications for the use of the multiple linear logistic model in other fields. Therefore, a cleaner, simpler example of the problem, illustrated in figure 30, is presented here. The four curves, *a*, *b*, *c* and *d* for both panels, A and B, were simulated with the simple linear logistic model, Eq. 37. For curve *a*, $\beta_0 = 1.5$, $\beta_1 = -2.0$; for curve *b*, $\beta_0 = 3.0$, $\beta_1 = -2.0$; for curve *c*, $\beta_0 = 1.5$, $\beta_1 = -0.1$; for curve *d*, $\beta_0 = 3.0$, $\beta_1 = -0.1$. In panel A, μ is plotted against agent dose on a common logarithmic scale; whereas, in panel B, the logit of μ is plotted against agent dose on a linear scale. For each of these curves, 45 points, indicated by symbols, were simulated, and then the points connected via the spline option in SigmaPlot 2.0 (Jandel Scientific, 1994). The combined data for curves *b* and *c* ($n = 90$) were assumed to represent the proportion of organisms remaining after treatment with agent 1 and agent 2, respectively. A sample size of 1000 was assumed for each of the 90 treatment groups. No binomial variation was introduced. This set of data was then fit with Eq. 38, a multiple linear logistic model with three estimable parameters, β_0, β_1, and β_2, but no β_{12} interaction parameter, with the LR program in BMDP (Dixon et al., 1990). This model assumes a common β_0 term, a β_1 term for agent 1, and a β_2 term for agent 2. The best fit estimates (± standard error) were: $\beta_0 = 1.93 \pm 0.013$; $\beta_1 = -1.36 \pm 0.016$; and $\beta_2 = -0.122 \pm 0.0015$. These parameter estimates were then used to simulate curves *e* and *f* in both panels (A) and (B).

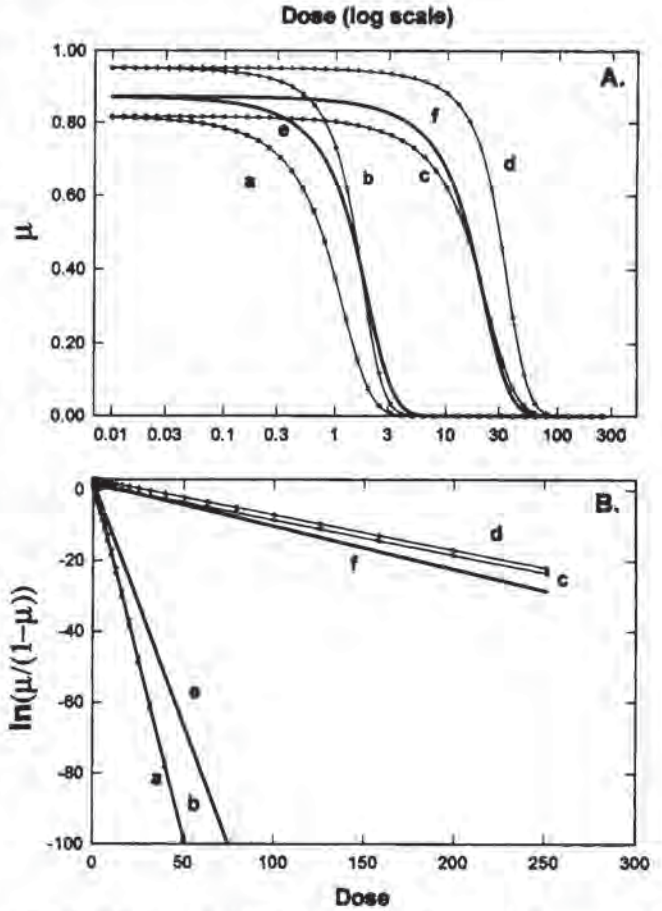


FIG. 30. Problems with use of logistic function for representing dose-response phenomena.

$$\mu = \frac{\exp(\beta_0 + \beta_1 D_1 + \beta_2 D_2)}{1 + \exp(\beta_0 + \beta_1 D_1 + \beta_2 D_2)} \qquad [38]$$

Note that in panel (A), the two members of each curve pair, *a* and *c*, *b* and *d*, and *e* and *f* share the same shape: they are parallel; they have the same dose-effect slope (on a logarithmic dose scale). For example, at every effect level (except $\mu = 1$ and $\mu = 0$), the dose for curve *c* is 20-fold higher than the corresponding dose on curve *a* (the ratio of their ID_{50}s). This is caused by the same β_0 term for each respective pair. The lateral separation of the curves for each pair is because of different β_1 terms. Because the ID_{50} is equal to $-\beta_0/\beta_1$, it is clear that a larger β_0 term will shift the concentration-effect curve to the right, and a larger-in-magnitude β_1 term will shift the curve to the left. Note that in panel B, that curve pairs *a* and *b*, and *c* and *d* consist of parallel lines. This is caused by the same β_1 terms for each respective pair. There are common y-intercepts for curve pairs *a* and *c*, *b* and *d*, and *e* and *f*, in panel B, but this cannot be visually detected on the scale with which the graph is drawn.

When Eq. 38 is fit to the combined data from curves *b* and *c*, there are only three parameters available to represent the information that was originally contained in four parameters, so compromises were necessary. Note that the estimated β_0 for the combined data, 1.93, is a compromise between the β_0 terms for the individual concentration-effect curves, 1.5 and 3.0. Also note that the concentration-effect slopes in panel (A) for curves *e* and *f* appear to be the same, but these curves are different from those of *a* and *c*, and of *b* and *d*. The estimated β_1 term for curve *f*, -0.122, is somewhat different from the β_1 term of curve *c*, -0.1; the estimated β_2 term for curve *e*, -1.36, is somewhat different from the β_1 term of curve *b*, -2.0. The ID_{50}s for curves *e* and *f*, 1.42 and 15.8, respectively, are close to those of curves, *b* and *c*, 1.5 and 15, respectively. Curves *e* and *f* seem to attempt to closely follow the data from curves *b* and *c*, but fail, because the information contained in four parameters cannot be expressed completely by three parameters.

The advantages and disadvantages of fitting 3-D combined-action concentration-effect surfaces to proportion data are essentially the same as listed for continuous data. However, as seen with the experience of the multivariate linear logistic model, one must be very careful about choosing an appropriate combined-action model.

VII. Overall Conclusions on Rival Approaches

Tables 5 and 6 summarize the characteristics of the 13 rival approaches for assessing combined-action for continuous data, and the three rival approaches for assessing combined-action for quantal data, respectively. In addition, they also provide a condensed summary of the conclusions of each analysis. For the originators of the 13 approaches for continuous data, there is about an equal division between those who have Loewe additivity as their null reference model and those that have Bliss independence as their null reference model. Only the method of Steel and Peckham (1979) and the method of Chou and Talalay (1984) use additional models, Eq. 20 and Eq. 18, respectively, as integral null reference models for their approaches.

With today's universal accessibility to powerful, inexpensive computers with useful software, there is no good reason for an analysis of combined-action data to lack a graphical component. All of the methods that require graphics and advanced statistical procedures have either already been implemented into stand-alone software packages or are "easily" implemented with standard general statistical and graphical software.

At first glance, the conclusions of the authors of the 13 different approaches seem to be quite varied. However, the common continuous data set was simulated with Eq. 5 to contain a small degree of Loewe synergism (true $\alpha = 0.5$), which corresponds in most regions of the 3-D concentration-effect surface to a small degree of Bliss antagonism. Methods 1, 2, 4, 5, 8, and 10 through 12b yielded conclusions consistent with their respective "no interaction" reference models. This was also true for method 3, the method of Valeriote and Lin (1975), which further divides Bliss antagonism into three subcategories, including "subadditivity." In addition, because the true combined-action was between Loewe additivity and Bliss independence, ideal data would fall into the additivity envelope of method 6, that of Steel and Peckham (1979), and thus the conclusion of "additivity" for this approach is also consistent. The Bliss independence surface approach failed to detect the small amount of Bliss antagonism. Of the 13 different approaches, only the method of Chou and Talalay (1984) gave a conclusion opposite to the one expected, based on its respective null reference model(s). This is because of artifacts inherent in the calculation of the *CI* vs. *fa* plot.

The three approaches to the analysis of combined-action for quantal data listed in table 6 all share Loewe additivity as the null reference model. The method of Gessner (1974) is somewhat conservative and just missed the correct conclusion of a small degree of Loewe synergism. Not surprisingly, our flagship model that was used in the simulation of the common proportion data set, table 4, fit the data well, but just barely detected the small degree of synergism (true $\alpha = 1$), just above the noise level of the data. The multivariate linear logistic model arrived at the wrong conclusion, because it could not mold itself well to the data.

VIII. Experimental Design

The main decisions that must be made regarding experimental design are: (*a*) where to choose the concentrations, (*b*) numbers of replicates, and (*c*) numbers of experiments. These seemingly simple questions have spawned many full careers for statisticians, who have delved deeply into them to reveal their inherent complexity. The adoption of a response surface paradigm for the assessment of combined-action of agents facilitates the understanding of formal statistical experimental design. First, the experimenter must decide whether he is in an exploratory or a confirmatory mode. Screening experiments (exploratory mode) should first include, for each agent individually, agent concentrations that span the anticipated response region. Logarithmic spacing of the concentrations over a thousand-fold to a million-fold range is probably necessary, depending upon the previ-

TABLE 5

Comparison of conclusions from the application to the same simulated data set (representative example of continuous data from pure small synergism with 10% relative error), columns 2 through 4 of Table 3, of 13 rival approaches for assessing the nature and intensity of agent combined-action

Approach	Null "no interaction" reference model*	Software availability†	Graphical approach?	"Advanced" statistical approach?‡	Long conclusion	Short conclusion§
1. Isobologram by hand	LADD	NN	N	N	No conclusion if IC_{50} values are used. Loewe synergism if IC_{80} values are used.	LSYN
2. Fractional Product method (Webb, 1963)	BIND	NN	N	N	Overall Bliss antagonism, possible Bliss synergism at high concentrations.	BANT
3. Method of (Valeriote and Lin, 1975)	BIND	NN	N	N	Overall subadditivity, possible Bliss synergism at high concentrations.	SUB
4. Method of (Drewinko et al., 1976)	BIND	NN	N	N	Significant Bliss antagonism ($P < 0.05$).	BANT
5. Interaction index calculation (Berenbaum, 1978)	LADD	NN, YG	N	N	Overall Loewe synergism, possibly some Loewe antagonism.	LSYN
6. Method of Steel and Peckham (1979)	BIND, Eq. 20	YS	Y	N	Most of the combination points lie within the additivity envelope.	ADD
7. Median-effect method (Chou and Talalay, 1984)	LADD, Eq. 18	YS	Y	N	No conclusion on exclusivity. Strong Loewe antagonism, $fa < 0.2$. Weak Loewe synergism, $fa > 0.8$.	LANT and LSYN
8. Loewe Additivity surface method (Berenbaum, 1985)	LADD	YG	Y	Y	Overall Loewe synergism, with the intensity increasing at higher concentrations.	LSYN
9. Bliss independence surface method	BIND	YG	Y	Y	Overall Bliss independence.	BIND
10. MacSynergy (Prichard and Shipman, 1990)	BIND	YS	Y	N	Overall Bliss antagonism, possible small Bliss synergism at $D_1 = 5–10$, $D_2 = 1$.	BANT
11. 3-D Spline fitting, contours (Sühnel, 1990)	LADD	YG	Y	Y	Overall small Loewe synergism, possible Loewe antagonism at a few effect levels.	LSYN
12a. Parametric response surface approach (Greco et al, 1990)	LADD	YG	Y	Y	Small, but significant ($P < 0.05$) overall Loewe synergism, $\alpha = 0.519 \pm 0.11$.	LSYN
12b. Parametric response surface approach (Weinstein et al, 1990)	LADD	YS	Y	Y	Significant ($P < 0.05$) overall Loewe synergism.	LSYN

* The null reference abbreviations are: LADD (Loewe additivity), BIND (Bliss independence).

† The abbreviations are: NN (not needed), YS (yes, a specific software package is available), YG (yes, in general, the user must implement the approach with common statistical and/or graphical software packages).

‡ Advanced statistical approaches, which all require sophisticated numerical algorithms, include spline-fitting, nonlinear regression and maximum likelihood estimation.

§ The short conclusion abbreviations are: LSYN (Loewe synergism), BANT (Bliss antagonism), ADD (additivity) defined by Steel and Peckham (1979), and SUB (subadditivity) defined by Valeriote and Lin (1975).

TABLE 6

Comparison of conclusions from the application to the same simulated data set (representative example of data from pure small synergism with binomial variation, Table 4), of three rival approaches for assessing the nature and intensity of agent combined-action

Approach	Null "no interaction" reference model*	Software availability	Graphical approach?	"Advanced" statistical approach	Long conclusion	Short conclusion
1. Method of (Gessner, 1974)	LADD	YS, YG	Y	N	Loewe additivity is claimed, but with a hint of small Loewe synergism.	LADD
2a. Parametric response surface approach (Greco and Lawrence, 1988)	LADD	YG	Y	Y	Small, borderline significant Loewe synergism ($P < 0.05$). $\alpha = 0.903 \pm 0.46$	LSYN
2b. Parametric response surface approach, multivariate logistic model (e.g., Carter et al, 1988)	LADD	YS	Y	Y	Significant Loewe antagonism ($P < 0.05$).	LANT

* The abbreviations used throughout Table 6 are the same as used in Table 5.

ous knowledge of the researcher about the concentration-effect behavior of the compound. After the individual agent concentration-effect curves are well characterized, a combination experiment should be conducted that repeats the single agent data points and which includes a set of combination points. Either a full factorial (checkerboard) design as suggested by Prichard and Shipman (1990), or a single ray (fixed-ratio) design, or a multiple ray design, all with logarithmically spaced concentrations, might be appropriate. If a complex 3-D concentration-effect surface is anticipated, then the entire interesting region of agent 1 and agent 2 concentrations should be sampled, either with a checkerboard or multiple ray design. However, if a well behaved 3-D concentration-effect surface is anticipated, and the specific combination being studied is only one of many candidates being screened, then a single ray may be sufficient. Composite designs consisting of a checkerboard and some rays might also be used. Of course, if the intended data analysis approach is firmly tied to a particular design, then that design will have to be used.

After the researcher has completed the analysis of the first mixture experiment in exploratory mode, he/she may want to switch to confirmatory mode. The repeat of the combination experiment may use the same design as in the exploratory experiment, but probably the knowledge gained from the first run will help to refine the design for the second run. If a complex 3-D concentration-effect surface was found in the exploratory experiment, then agent concentrations in the interesting regions of the surface should be accented in the confirmatory experiment. Increasing the numbers of replicates probably also will be necessary. If a simple 3-D concentration-effect surface was found in the exploratory experiment, i.e., one with pure Loewe synergism or Loewe antagonism, then a design that facilitates the estimation of parameters with the smallest variance might be appropriate. A single ray or a D-optimal design (Box and Lucas, 1959; Atkinson and Hunter, 1968; Silvey, 1980; Fedorov, 1972; Greco and Tung, 1991) might be indicated with many replicates.

There are many lettered-optimality criteria for experimental design. Atkinson and Donev (1992) present a recent comprehensive review. The D-optimality criterion has become popular for biological applications (e.g., Bezeau and Endrenyi, 1986; Greco et al., 1994). Reasons for its popularity include: (*a*) ease of application; (*b*) intuitiveness of its theoretical basis (For models nonlinear in the parameters, D-optimality minimizes the linear approximation of the volume of the joint confidence region of the parameters); (*c*) transformation of model parameters does not alter designs (Fedorov, 1972).

Interestingly, the number of design points in a D-optimal design is generally equal to the number of estimable parameters (Atkinson and Hunter, 1968). For example, if one assumes that Eq. 5, which contains 6 parameters, will adequately describe the 3-D combined-action concentration-effect curve, then a D-optimal design will include only six design points, with or without replicates. A description of our algorithms for calculating D-optimal designs for agent combination studies is included in Greco and Tung (1991) and Greco et al. (1993).

The D-optimal designs may, at first, seem to be very strange and potentially noninformative. For example, for the continuous common data set listed in table 2, which contains proportional error, the approximate D-optimal design based upon the ideal parameters ($Econ = 100$, $IC_{50,1} = 10$, $IC_{50,2} = 1$, $m_1 = -1$, $m_2 = -2$, $\alpha = 0.5$) is (point 1, $D_1 = 0$, $D_2 = 0$; point 2, $D_1 = 1{,}000$, $D_2 = 0$; point 3, $D_1 = 95$, $D_2 = 0$; point 4, $D_1 = 0$, $D_2 = 1000$; point 5, $D_1 = 0$, $D_2 = 3.08$, point 6, $D_1 = 86.4$, $D_2 = 8.73$). This D-optimal design is only approximate because the assumption of pure proportional error (constant coefficient of variation) will drive many of the design points to unrealistic infinite concentrations (Bezeau and Endrenyi, 1986). We have reduced unrealistically large concentrations to 1000. Even with this adjustment, the D-optimal design still seems to be uninformative. (By visually plotting the six D-optimal design points in figure 25, the reader will note that one point is at the very top of the concentration-effect surface and that the other five are at the bottom! None of the points lie in the middle region of the surface.) However, we have conducted Monte-Carlo simulations to verify that this type of D-optimal design results in the smallest variance for the six model parameters when compared with factorial and ray designs (Greco et al., 1994). We have also shown that the variance of the parameter estimates is approximately proportional to the reciprocal of the number of replicates. This type of frugal experimental design may have great potential for animal and human experiments, in which the experimental units are very dear.

The point at which the Loewe additivity model and the combined-action model are furthest apart in the vertical direction may be an important design point; this point may offer the maximum potential for discriminating between the two models (Mannervick, 1982). From figure 10(C), it was shown for our flagship model, with parameters ($Econ$ = 100, $IC_{50,1}$ = 1, $IC_{50,2}$ = 1, m_1 = -1, m_2 = -2, α = 5), that the largest vertical difference was near the point, ($IC_{50,1}$, $IC_{50,2}$). In contrast, figure 9 indicates that the largest horizontal difference between Loewe additivity and our combined-action model is at infinite concentrations of both agents. This implies that a pair of very large concentrations may be useful. These two design points, based upon maximum model differences, may be added to other designs discussed above.

Formal statistical experimental design often includes an interesting paradox: in order to design an experiment well, you have to know the final answer well. However, if you knew the final answer well, then you would not have to conduct the experiment. This paradox is solved with sequential experimentation; each experiment in a sequence provides better information for the planning of the subsequent experiment.

IX. General Proposed Paradigm

Readers of this review may not be particularly happy at this point. They may have become enlightened on the subject of combined-action after following the discussion of the different 3-D and 2-D representations of this phenomena. They may have carefully read the descriptions of the application of 13 rival approaches for assessing combined-action for continuous data, and of 3 rival approaches for quantal data. They may have digested and evaluated the long list of advantages and disadvantages of each approach. They may now have a greater appreciation of the similarities and differences among the rival approaches reviewed in this paper. Finally, they may have developed an understanding of the fundamental importance of mathematical models in the description and evaluation of complex systems. However, it is probably not at all clear how to actually proceed with the practical analysis of a data set from an experiment of combined-action.

We recommend the following general approach. Before the combined-action experiment is conducted, the concentration-effect curves for the individual agents should be characterized well. Data for a combination experiment can then be generated from either a factorial design, from a fixed-ratio (ray) design, from a D-optimal design, from a model discrimination design, or some combination of the four. The numbers and distribution of different rows and columns in the factorial design, the numbers and distributions of rays in the fixed-ratio design, and the numbers of replicates, will depend upon the importance of the anticipated result, the cost of each experimental unit, and the degree of ignorance of the shape of the full 3-D concentration-effect surface.

The overall best initial data analysis, which will work with almost any conceivable, reasonable design, should include a combination of approaches V.H., the method of Berenbaum (1985), and V.I., the Bliss independence response surface approach. First, a logical Loewe additivity model should be fit, with an appropriate curve-fitting technique, to the data for agent 1 alone and agent 2 alone. Nonlinear regression should be used to fit models to continuous data, and maximum likelihood procedures used to fit models to quantal data. The 3-D Loewe additivity predicted surface should be shown in 3-D. Then sprinkle the raw data points on the same graph, and note the position of the points relative to the surface, such as was done in figure 18(A). Then construct the Bliss independence surface and sprinkle the raw data points, such as was done in figure 18(B). Combining Loewe additivity and Bliss independence surfaces on the same 3-D graph may be useful. Also, various 2-D representations of the 3-D surfaces, such as isobolograms, and families of 2-D concentration-effect curves, with accompanying data points, may be useful. A confidence envelope, adapted from suggestions of Carter et al. (1986, 1988), around the two surfaces might be used to discriminate between true departures from the null reference models and random variation. Note that our suggested approach has the flavor of the "additivity envelope" method of Steel and Peckham (1979), but the correct model for Loewe additivity is used to define one of the boundaries, instead of Eq. 20. Only in rare cases will it be difficult to find appropriate concentration-effect models to fit the concentration-effect data for the individual agents.

After this initial analysis, a decision should be made whether to derive and fit a full appropriate combined-action concentration-effect model to all of the experimental data simultaneously or to accept the initial analysis as the final answer. In many cases, it will be fruitful to complete this last step. The final summary statistics should include uncertainty measures around the final parameter estimates, confidence envelopes around the fitted surface, overall goodness of fit statistics, residual analyses, and sets of 3-D and 2-D graphs. These sets of graphs may include the 3-D combined-action concentration-effect surface along with the raw data, such as figure 22, 3-D difference plots such as figure 10, 3-D combination index plots such as figure 9, 2-D isobolograms such as figure 23, 2-D families of concentration-effect curves, such as figures 24 and 25, plus any other informative graphical representations. Physical 3-D models of combined-action concentration-effect surfaces made with LEGO bricks (LEGO Systems Inc., Enfield, CT) (Greco, 1991) or other materials can accent important results.

To the best of our knowledge, a software package dedicated exclusively to this whole composite approach does not as yet exist. However, many general nonlinear regression packages, which allow the coding of a one-dimensional root finder for dealing with models in unclosed form, and with accompanying graphics capabilities, could be used to implement this approach. Such packages available for microcomputers include: PC-NONLIN (Statistical Consultants Inc., 1986), SAS (SAS Institute Inc., 1987), MLAB (Civilized Software, Inc., 1991), GAUSS (Aptech Systems Inc., 1991), and IMSL (IMSL, 1989). There are many more packages available for UNIX workstations, minicomputers, and mainframe computers with adequate capabilities to implement this full approach. Our group is currently developing an implementation of the full approach, which has been designed to work under the MicroSoft Windows operating system.

Several critical areas for future research and development in the field of the assessment of combined-action were pointed out in this review article:

(*a*) the relationship between empirical models of combined-action, and mechanistic theoretical models of biochemical and physiological systems should be explored.

(*b*) a library of combined-action models should be derived, collected, evaluated, and critically compared.

(*c*) the impact of using different experimental designs, especially D-optimal designs, should be evaluated, both from theoretical and practical perspectives.

(*d*) user-friendly, inexpensive computer software should be developed to facilitate the paradigm of experimental design and data analysis approaches described above.

X. Appendix A. Derivation of a Model for Two Mutually Nonexclusive Noncompetitive Inhibitors for a Second Order System

A. Motivation

The concepts of Bliss independence and mutual nonexclusivity, at first glance, seem to be the same. Equivalent general forms for the classical Bliss independence model are Eqs. 11 and 14, in which fu_1, fu_2, and fu_{12} are the fractions of possible response for drug 1, drug 2, and the combination (e.g., % survival, %control) unaffected (Chou and Talalay, 1981, 1984), and fa_1, fa_2, and fa_{12} are the fractions of possible response affected (e.g., % dead, % inhibition) [$fa(= 1 - fu)$]. For the common case in which each drug individually follows the Hill concentration-effect model, Eq. 2, (equivalent to the median-effect equation of Chou and Talalay, Eq. 24) the appropriate specific Bliss independence model would be Eq. 12 ($fu = E/Econ$).

$$fu_{12} = fu_1 fu_2 \qquad [11]$$

$$fa_{12} = fa_1 + fa_2 - fa_1 fa_2 \qquad [14]$$

$$E = \frac{Econ\left(\dfrac{D}{IC_{50}}\right)^m}{1 + \left(\dfrac{D}{IC_{50}}\right)^m} \qquad [2]$$

$$\frac{fa}{fu} = \left(\frac{D}{Dm}\right)^m \qquad [24]$$

$$E = \frac{Econ\left(\dfrac{D_1}{IC_{50,1}}\right)^{m_1}\left(\dfrac{D_2}{IC_{50,2}}\right)^{m_2}}{\left(1 + \left(\dfrac{D_1}{IC_{50,1}}\right)^{m_1}\right)\left(1 + \left(\dfrac{D_2}{IC_{50,2}}\right)^{m_2}\right)} \qquad [12]$$

However, the mutually nonexclusive model of Chou and Talalay (1981, 1984), Eq. 18, is not equivalent to the Bliss independence model, except under the restrictive condition that the slope parameter, m, is equal to 1 (or to -1 by our convention of monotonically decreasing concentration-effect curves). Eq. 19 is a specific nonlinear form of Eq. 18. (Note that Eq. 19 is equivalent to our flagship interaction model, Eq. 5, with $m_1 = m_2 = m$, and $\alpha = 1$.) Chou and Talalay (1984) stressed this difference between the Bliss independence model and their mutually nonexclusive model and concluded that the Bliss independence model is not appropriate for higher order systems ($m > 1$).

$$\left(\frac{fa_{12}}{fu_{12}}\right)^{1/m} = \left(\frac{fa_1}{fu_1}\right)^{1/m} + \left(\frac{fa_2}{fu_2}\right)^{1/m} + \left(\frac{fa_1 fa_2}{fu_1 fu_2}\right)^{1/m} = \frac{D_1}{Dm_1} + \frac{D_2}{Dm_2} + \frac{D_1 D_2}{Dm_1 Dm_2} \qquad [18]$$

$$E = \frac{Econ\left(\dfrac{D_1}{IC_{50,1}} + \dfrac{D_2}{IC_{50,2}} + \dfrac{D_1 D_2}{IC_{50,1} IC_{50,2}}\right)^m}{1 + \left(\dfrac{D_1}{IC_{50,1}} + \dfrac{D_2}{IC_{50,2}} + \dfrac{D_1 D_2}{IC_{50,1} IC_{50,2}}\right)^m} \qquad [19]$$

We certainly agree that Eqs. 12 and 19 are not equivalent. It should be noted that the derivation of the mutually nonexclusive model (Chou and Talalay, 1981) was for multiple mutually nonexclusive reversible inhibitors of a single enzyme, in which the slope parameter, m, is the integral number of binding sites on the enzyme for each inhibitor, yet the application of the model has been mainly to much more complex systems, such as cell cultures and batches of whole organisms, in which the nonintegral slope parameter, m, is related to the width of the tolerance distribution of the sensitivities of the cells or organisms to the agent. It might be argued that the difference between Eqs. 12 and 19 is caused by their differences in origin. However, we will show below that the primary reason that Bliss independence and mutually nonexclusivity are not equivalent is that the mutually nonexclusive model of Chou and Talalay (1981) was not properly derived.

B. Elements of the Derivation of the Mutually Nonexclusive Model for Higher Order Systems from Chou and Talalay (1981)

To keep confusion to a minimum, we will use fi and fv for the fractional inhibition and fractional velocity, respectively, which are slightly different from the variable symbols included in Chou and Talalay (1981). Also, instead of using Chou and Talalay's exact general equations for any number of enzyme inhibitors, we will list specific equations for sets of two inhibitors. We will designate Chou and Talalay's equations with a CT prefix, and use the equation number from Chou and Talalay (1981).

The key suspicious step in the derivation of the mutually nonexclusive model, Eq. CT22, appears on page 211 of Chou and Talalay (1981). It is stated:

Let us assume that m molecules of each of two mutually nonexclusive inhibitors bind to one molecule of enzyme. By analogy to Eqn (CT17) and addition of the term for nonexclusivity [Eqn(CT21)] we obtain:

$$\left[\frac{fi_{12}}{fv_{12}}\right]^{1/m} = \left[\frac{fi_1}{fv_1}\right]^{1/m} + \left[\frac{fi_2}{fv_2}\right]^{1/m} + \left[\frac{fi_1 fi_2}{fv_1 fv_2}\right]^{1/m}$$

or

$$fv_{12} = \frac{1}{1 + \left\{\left[\frac{fi_1}{fv_1}\right]^{1/m} + \left[\frac{fi_2}{fv_2}\right]^{1/m} + \left[\frac{fi_1 fi_2}{fv_1 fv_2}\right]^{1/m}\right\}^m} \quad \text{[CT22]}$$

$$= \frac{1}{1 + \left[\frac{I_1}{I_{50,1}} + \frac{I_2}{I_{50,2}} + \frac{I_1 I_2}{I_{50,1} I_{50,2}}\right]^m}$$

It is our view that merely stating, "*by analogy to Eqn(CT17) and addition of the term for nonexclusivity [EQN(CT21)], we obtain:*" does not constitute a convincing derivation. Eq. CT17, or the equivalent, Eq. CT18, that for a mutually exclusive system was derived by combining Eq. CT11, the general equation for mutual exclusivity for multiple inhibitors in a first order system with Eq. CT12, the general median-effect or Hill equation for inhibition of higher order kinetic systems by a single inhibitor.

$$\frac{fi_{12}}{fv_{12}} = \frac{fi_1}{fv_1} + \frac{fi_2}{fv_2} = \frac{I_1}{I_{50,1}} + \frac{I_2}{I_{50,2}} \quad \text{[CT11]}$$

$$\frac{fi}{fv} = \left[\frac{I}{I_{50}}\right]^m \quad \text{[CT12]}$$

$$\frac{fi_{12}}{fv_{12}} = \left[\frac{I_1}{I_{50,1}} + \frac{I_2}{I_{50,2}}\right]^m \quad \text{[CT17]}$$

which can be rewritten:

$$fv_{12} = \frac{1}{1 + \left[\frac{I_1}{I_{50,1}} + \frac{I_2}{I_{50,2}}\right]^m} \quad \text{[CT18]}$$

Even for the derivation of Eq. CT17, that for mutual exclusivity, it is not entirely apparent to us how to properly combine Eqs. CT11 and CT12. However, via two other derivations not provided here, one based on enzyme kinetics and another based on the ideas of Berenbaum (1985) and provided in Appendix A of Greco et al. (1990), we verified that Eq. CT17, that for mutual exclusivity, is correct.

Thus, the derivation of the mutually nonexclusive model for two enzyme inhibitors provided by Chou and Talalay (1981) is weak, incomplete, and suspicious. In order to settle the matter, we provide below a complete derivation for the case of two mutually nonexclusive, noncompetitive inhibitors of a single enzyme. We use the same restrictive assumption used by Chou and Talalay (1981) and also used in the derivation by Hill (1910) that, for each inhibitor, which has two identical binding sites on the enzyme, both of the two inhibitor molecules bind to the enzyme in one step. It should be emphasized that our goal is not to derive an alternate model for mutual nonexclusivity to be used by the biomedical community but rather to show that the Chou and Talalay model was not derived correctly. We therefore provide this one counterexample, for two mutually nonexclusive, noncompetitive inhibitors, to refute the general model for mutual nonexclusivity of Chou and Talalay (1981).

C. Assumptions of the Derivation of the Model for Mutual Nonexclusivity for Two Noncompetitive Higher Order inhibitors

1. The enzyme (E) has one active site where one substrate molecule (S) may bind.

2. In addition to the active site for the substrate, there are two binding sites for inhibitor 1 and two other binding sites for inhibitor 2. Any occupation of an inhibitor site will prevent the substrate from being converted to product.

3. Both inhibitor 1 and 2 are noncompetitive with the substrate; 2 molecules of inhibitor 1 plus two molecules of inhibitor 2 may simultaneously bind to the enzyme, whether the substrate has occupied the active site or not.

4. The affinity of inhibitor 1 for the enzyme, and the affinity of inhibitor 2 for the enzyme, is unaffected by occupation of the active site by the substrate; thus, we have classical or pure noncompetitive inhibition.

5. The binding of inhibitor 1, I_1, to its binding sites does not influence the binding of inhibitor 2, I_2, to its binding sites, and vice versa.

6. When I_1 binds, two molecules bind at once; the same for I_2. [This is the critical controversial Hill assumption, which was also made by Chou and Talalay (1981) in the derivation of the median effect equation for a single inhibitor.] In other words, the concentrations of enzyme species, E, ES, EI_1I_1, EI_2I_2, $EI_1I_1I_2I_2$, ESI_1I_1, ESI_2I_2, $ESI_1I_1I_2I_2$, exist; but EI_1, EI_2, ESI_1, ESI_2, EI_1I_2, $EI_1I_2I_2$, $EI_1I_1I_2$, ESI_1I_2, $ESI_1I_2I_2$, $ESI_1I_1I_2$ are negligible and will be assumed to not exist.

D. Derivation

1. The general rules for deriving enzyme kinetic rate equations from Segel (1975) are used.

2. The enzyme velocity (v) rate equation is written in terms of the rate constant for the formation of product (k_p) and the enzyme-substrate complex concentration ([ES]):

$$v = k_p[\mathrm{ES}] \qquad [A1]$$

3. The left side of the velocity equation is divided by the concentration of total enzyme, $[E_t]$, and the right side is divided by the equivalent sum of the concentrations of all non-negligible enzyme species: (Note: The denominators of Equations A2, A3 and A7 are too wide to fit easily into an equation in one column of a journal page. Therefore, each denominator has been defined by the terms, *DENOMA2*, *DENOMA3*, *DENOMA7*, respectively):

$$DENOMA2 = [E] + [ES] + [EI_1I_1] + [EI_2I_2] + [EI_1I_1I_2I_2] + [ESI_1I_1] + ESI_2I_2] + [ESI_1I_1I_2I_2]$$

$$\frac{v}{[E_t]} = \frac{k_p[\mathrm{ES}]}{DENOMA2} \qquad [A2]$$

4. Concentrations of each species are expressed in terms of [E]. The term for any given complex is composed of a numerator and a denominator. The numerator is the product of the concentrations of all ligands in the complex. The denominator is the product of all dissociation constants between the complex and free enzyme, E. Also, let the maximum enzyme velocity, $Vmax = k_p\,[E_t]$.

$$DENOMA3 = 1 + \left[\frac{S}{Ks}\right] + \left[\frac{I_1}{Ki_1}\right]^2 + \left[\frac{I_2}{Ki_2}\right]^2 + \left[\frac{I_1}{Ki_1}\right]^2\left[\frac{I_2}{Ki_2}\right]^2 + \left[\frac{S}{Ks}\right]\left[\frac{I_1}{Ki_1}\right]^2 + \left[\frac{S}{Ks}\right]\left[\frac{I_2}{Ki_2}\right]^2 + \left[\frac{S}{Ks}\right]\left[\frac{I_1}{Ki_1}\right]^2\left[\frac{I_2}{Ki_2}\right]^2$$

$$\frac{v}{Vmax} = \frac{S/Ks}{DENOMA3} \qquad [A3]$$

5. For a noncompetitive inhibitor, $I_{50} = Ki$ (Chou, 1974). Therefore, all *Kis* are replaced with I_{50}s. In addition, Eq. A3 is simplified to Eq. A4.

$$v = \frac{Vmax[S/Ks]/[1 + S/Ks]}{1 + \left[\frac{I_1}{I_{50,1}}\right]^2 + \left[\frac{I_2}{I_{50,2}}\right]^2 + \left[\frac{I_1}{I_{50,1}}\right]^2\left[\frac{I_2}{I_{50,2}}\right]^2} \qquad [A4]$$

6. The fractional velocity, *fv*, is equal to the ratio of the inhibited velocity, Eq. A4, divided by the uninhibited velocity, equal to $[Vmax\ S]/[Ks + S]$. After this operation and some simplification, Eq. A5 is the result.

$$fv = \frac{1}{1 + \left[\frac{I_1}{I_{50,1}}\right]^2 + \left[\frac{I_2}{I_{50,2}}\right]^2 + \left[\frac{I_1}{I_{50,1}}\right]^2\left[\frac{I_2}{I_{50,2}}\right]^2} \qquad [A5]$$

Eq. A5 can be written in an equivalent form, Eq. A6.

$$fv = \left\{\frac{1}{1 + \left[\frac{I_1}{I_{50,1}}\right]^2}\right\}\left\{\frac{1}{1 + \left[\frac{I_2}{I_{50,2}}\right]^2}\right\} \qquad [A6]$$

7. Note that Eq. A5 is not equivalent to the mutually nonexclusive model of Chou and Talalay (1981) for the case of second order inhibitors ($m = 2$). Rather, Eq. A5 and its equivalent, Eq. A6 is exactly equivalent to the Bliss independence model, Eq. 11, for two second order inhibitors. Thus, a complete specific derivation for the case of two mutually nonexclusive, second order, noncompetitive enzyme inhibitors, which follows the general but incomplete derivation provided by Chou and Talalay (1981), yields an equation inconsistent with their final model, Eq. CT22, but consistent with the Bliss independence model, Eq. 11.

E. Possible Rationalization of the Mutually Nonexclusive Model of Chou and Talalay (1981)

1. The expansion of the mutually nonexclusive model of Chou and Talalay (1981), Eq. CT22, for the case of

$m = 2$, yields Eq. A7.

$$DENOMA7 = 1 + \left[\frac{I_1}{I_{50,1}}\right]^2 + \left[\frac{I_2}{I_{50,2}}\right]^2 + \left[\frac{I_1}{I_{50,1}}\right]^2\left[\frac{I_2}{I_{50,2}}\right]^2 + 2\left[\frac{I_1}{I_{50,1}}\right]\left[\frac{I_2}{I_{50,2}}\right] + 2\left[\frac{I_1}{I_{50,1}}\right]^2\left[\frac{I_2}{I_{50,2}}\right] + 2\left[\frac{I_1}{I_{50,1}}\right]\left[\frac{I_2}{I_{50,2}}\right]^2$$

$$fv = \frac{1}{DENOMA7} \qquad [A7]$$

2. The difference between this expansion of the mutually nonexclusive model of Chou and Talalay (1981), Eq. A7, and the mutually nonexclusive model derived above, Eq. A5, which is equivalent to Bliss independence, is the additional three right-hand terms in the denominator. These three terms imply the existence of six additional enzyme species—2 EI_1I_2, 2 $EI_1I_2I_2$, 2 $EI_1I_1I_2$, 2 ESI_1I_2, 2 $ESI_1I_2I_2$, and 2 $ESI_1I_1I_2$—that we initially assumed were negligible and did not exist. This stems from the key Hill assumption that when and if an inhibitor binds, either I_1 or I_2, two molecules of that inhibitor bind at once. Possibly, one might be willing to get rid of this assumption, and replace it with a less restrictive assumption such as:

EI_1, EI_2, ESI_1, ESI_2 are all negligible, but enzyme forms that contain at least two inhibitor molecules, possibly a mixture of the two inhibitors, including EI_1I_2, $EI_1I_2I_2$, $EI_1I_1I_2$, ESI_1I_2, $ESI_1I_2I_2$, and $ESI_1I_1I_2$, are not negligible.

If so, then the mutually nonexclusive model of Chou and Talalay (1981) would have a firmer theoretical basis. However, it is unlikely that an equation derived from a set of very unusual assumptions, for the rare case of two mutually nonexclusive higher order inhibitors of a single enzyme, would have general utility for modeling concentration-effect phenomena from a wide spectrum of complex agent interaction systems.

XI. Appendix B: Problems with the Use of the Median Effect Plot and Combination Index Calculations to Assess Drug Interactions

Both the inherent nonlinear nature of the median effect plot and the incorrect calculation of the combination index (*CI*), for the case of mutual nonexclusivity, contribute to incorrect artifactual conclusions concerning synergism and antagonism, when applying the method of Chou and Talalay (1984) to real laboratory data. In addition, the median effect plot for drug combination data for mutually exclusive drugs showing synergism or antagonism will also be nonlinear. The extent, origins, and impact of these problems are illustrated by the simulation shown in figures B1, B2, B3, in table B1, and the following narrative.

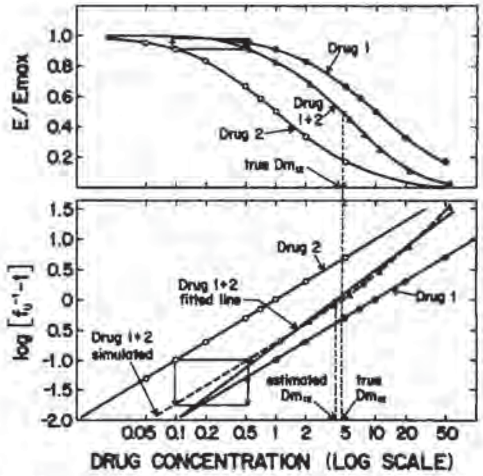


FIG. B1. Upper panel: Data points plotted from table B1. The data points and the curves connecting the points were simulated using Eq. B1, that for mutual nonexclusivity, with $Dm_1 = 10$, $Dm_2 = 1$, $m = 1$, and $R = 10$. The Y-axis is *E/Emax* or *fu*; the X-axis is the sum of drug 1 and drug 2 concentrations on a logarithmic scale. Lower panel: The three median-effect lines were made by separately fitting each of the three subsets of data with unweighted linear regression. The curved, dashed line is the median-effect curve for the combination of drug 1 + 2 simulated from Eq. B1. The rectangular boxes in each panel represent equivalent ranges of fractional effect. The arrows on the side of the boxes indicate the direction of decreasing *fa* (increasing *fu*).

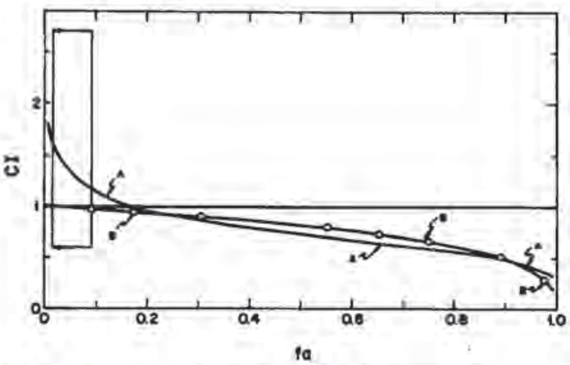


FIG. B3. Mutually exclusive *CI* vs. *fa* plots for the data from table B1 and figure B1. Curve A was generated in the exact way suggested by Chou and Talalay (1984) and included in the commercially available program (Chou and Chou, 1987); i.e., by estimating Dm_1, m_1, Dm_2, m_2, Dm_{12}, m_{12} with unweighted linear regression as in the lower panel of figure B1, and then plugging these values into Eq. 25 to calculate *CI* for a range of *fa* values. Curve B was generated by calculating Dm_{12} and m_{12} with Eqs. B2 through B4, from the original (same as estimated) values, $Dm_1 = 10$, $Dm_2 = 1$, $m_1 = m_2 = 1$, and then plugging these values into Eq. 25. The box represents a range of fractional effects equivalent to the boxes in figure B1, with the arrows of the box indicating the direction of decreasing *fa*. The open data points represent the eight combination points, each calculated with the *CI* formula for the mutually exclusive assumption for the raw data itself, Eq. 27.

A. *Nonlinear Nature of the Median Effect Plot for Mutual Nonexclusivity*

The median effect plot for mutually nonexclusive drugs is inherently nonlinear. This was shown originally by Chou and Talalay (1981) in figure 2 of their paper. Therefore, the estimation of Dm_{12} and m_{12} via simple linear regression can never be correct. The data points in

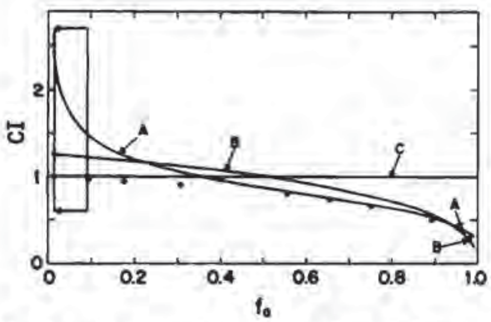


FIG. B2. Mutually nonexclusive CI vs. fa plots for the data from table B1 and figure B1. Curve A was generated in the exact way suggested by Chou and Talalay (1984) and included in the commercially available program (Chou and Chou, 1987); i.e., by estimating Dm_1, m_1, Dm_2, m_2, Dm_{12}, m_{12} with unweighted linear regression as in the lower panel of figure B1, and then plugging these values into Eq. 26 to calculate CI for a range of fa values. Curve B was generated by calculating Dm_{12} and m_{12} with Eqs. B2 through B4, from the original (same as estimated) values, $Dm_1 = 10$, $Dm_2 = 1$, $m_1 = m_2 = 1$, and then plugging these values into Eq. 26. Curve C, $CI = 1$, was generated by calculating Dm_{12} and m_{12} with Eqs. B2 through B4, but then using Eq. B5 for the CI calculation. The box represents a range of fractional effects equivalent to the boxes in figure B1, with the arrows of the box indicating the direction of decreasing fa. The open data points represent the eight combination points, each calculated with the CI formula for the mutually exclusive assumption for the raw data itself, Eq. 27.

figure B1 and table B1 were simulated by using Eq. B1, that for mutual nonexclusivity, and using $Dm_1 = 10$, $Dm_2 = 1$, $m = 1$, and $R = 10$. (Here, R is the ratio of concentrations of D_1:D_2). The data consists of 24 simulated data points, 8 for drug 1 alone, 8 for drug 2 alone, and 8 for the combination of drug 1+2 in a 10:1 constant ratio. Four significant figures were retained through all calculations to eliminate any appreciable errors in the simulated data.

$$\left[\frac{fa_{12}}{fu_{12}}\right]^{1/m} = \left[fu_{12}^{-1} - 1\right]^{1/m} = \frac{D_1}{Dm_1} + \frac{D_2}{Dm_2} + \frac{D_1 D_2}{Dm_1 Dm_2} \qquad \text{[B1]}$$

In the upper panel of figure B1, the three concentration-effect curves were simulated directly with Eq. B1. The data points in figure B1 correspond to the 24 simulated points in table B1. In the lower panel, the three median effect straight lines were made by separately fitting each of the three sets of data with unweighted linear regression. The curved, dashed line is the nonlinear median effect curve for the combination of drug 1+2 simulated from Eq. B1. The rectangular boxes in each panel represent equivalent ranges of effect. The arrows on the sides of the boxes indicate the direction of decreasing fa (from $fa = 0.091$ to 0.017). The parameters estimated from the three linear regression lines were: $Dm_1 = 10.0$, $m_1 = 1.00$, $Dm_2 = 1.00$, $m_2 = 1.00$, $Dm_{12} = 4.04$, $m_{12} = 1.24$. The correct Dm_{12} calculated from Eq. B2 was 4.56. The correct m_{12} calculated from Eqs. B3 and B4, which are fa-dependent, increased from 1.04 at $fa = 0.01$ to 1.49 at $fa = 0.99$. (The derivations of Eqs. B2 through B4, for the restricted case of $m_1 = m_2 = m$, are not included here but can be requested from W. R. Greco.) Note the vertical dashed line in figure B1, which shows the alignment of the true Dm_{12} value. Also note the small displacement of the estimated Dm_{12} value from the true Dm_{12}. It is the approximation of the varying m_{12} by a constant m_{12} estimated from the median effect linear regression, which is responsible for most of the mismatch between the true median effect nonlinear curve and the approximate straight line. (Note: The numerators of Equations B2 and B4 are too wide to fit easily into an equation in one column of a journal page. Therefore, each numerator has been defined by the terms *NUMB2* and *NUMB4*, respectively):

$$NUMB2 = -\left[\frac{RDm_2 + Dm_1}{(1+R)Dm_1Dm_2}\right] + \sqrt{\left[\frac{RDm_2 + Dm_1}{(1+R)Dm_1Dm_2}\right]^2 + \left[\frac{4R}{(1+R)^2Dm_1Dm_2}\right]}$$

$$Dm_{12} = \frac{NUMB2}{\left[\dfrac{2R}{(1+R)^2Dm_1Dm_2}\right]} \qquad \text{[B2]}$$

$$m_{12} = \frac{m\log\left[\dfrac{[RDm_2 + Dm_1]Z + RZ^2}{Dm_1Dm_2}\right]}{\log\left[\dfrac{Z(1+R)}{Dm_{12}}\right]} \qquad \text{[B3]}$$

where:

$$NUMB4 = \left[\frac{RDm_2 + Dm_1}{Dm_1Dm_2}\right] + \sqrt{\left[\frac{RDm_2 + Dm_1}{Dm_1Dm_2}\right]^2 + \left[\frac{4R}{Dm_1Dm_2}\right]\left[\frac{fa_{12}}{1-fa_{12}}\right]^{1/m}}$$

$$Z = \frac{NUMB4}{-2\left[\dfrac{R}{Dm_1Dm_2}\right]} \qquad \text{[B4]}$$

Figure B2 is a mutually nonexclusive CI vs. fa plot for the data in table B1 and figure B1. Curve A in figure B2 was calculated as suggested by Chou and Talalay (1984), from the three straight median effect lines in figure B1, using the formula, Eq. 26, incorporated into the commercial software package, Dose-Effect Analysis with Microcomputers (Chou and Chou, 1987). The interested reader should be able to reproduce this curve by plugging the 24 data points listed in table B1 into the commercial software package. Like many real examples from the literature, the standard CI vs. fa plot, curve A, crosses the additivity, $CI = 1$ line. The conclusion from

TABLE B1
*Simulated data for mutual nonexclusivity examination**

D_1	D_2	fu	fa	$\log[fu^{-1} - 1]$
0.5	0	0.9524	0.04762	−1.301
1	0	0.9091	0.09091	−1.000
2	0	0.8333	0.1667	−0.6989
5	0	0.6667	0.3333	−0.3011
7	0	0.5882	0.4118	−0.1548
10	0	0.5000	0.5000	0.0000
20	0	0.3333	0.6667	0.3011
50	0	0.1667	0.8333	0.6989
0	0.05	0.9524	0.04762	−1.301
0	0.1	0.9091	0.09091	−1.000
0	0.2	0.8333	0.1667	−0.6989
0	0.5	0.6667	0.3333	−0.3011
0	0.7	0.5882	0.4118	−0.1548
0	1	0.5000	0.5000	0.0000
0	2	0.3333	0.6667	0.3011
0	5	0.1667	0.8333	0.6989
0.5	0.05	0.9070	0.0930	−0.9891
1	0.1	0.8264	0.1736	−0.6776
2	0.2	0.6944	0.3056	−0.3565
5	0.5	0.4444	0.5556	0.09699
7	0.7	0.3460	0.6540	0.2765
10	1	0.2500	0.7500	0.4771
20	2	0.1111	0.8889	0.9031
50	5	0.02778	0.9722	1.544

* The data was simulated using Eq. B1, that for mutual nonexclusivity, with $Dm_1 = 10$, $Dm_2 = 1$, $m = 1$, and $R = 10$. This is an ideal data set with no random errors added; any inexactness is caused by roundoff errors in the fourth significant figure.

Curve A is appreciable antagonism at low fractional effects and appreciable synergism at high fractional effects. However, the data in table B1 was simulated for pure, unadulterated, mutual nonexclusivity! The final CI vs. fa plot should be a straight, horizontal line at $CI =$ 1! Note the large box on the left-hand side of figure B2. This is the same box as was shown in figure B1, upper and lower panel, for a range of concentration-effect, except that its height has been magnified in the CI vs. fa plot. Thus, the difference between the true nonlinear median effect curve, and the approximate median effect straight line, has been magnified in the CI vs. fa plot.

$$CI = \frac{Dm_{12}\left[\frac{R}{R+1}\right]\left[\frac{fa}{1-fa}\right]^{1/m_{12}}}{Dm_1\left[\frac{fa}{1-fa}\right]^{1/m_1}} + \frac{Dm_{12}\left[\frac{1}{R+1}\right]\left[\frac{fa}{1-fa}\right]^{1/m_{12}}}{Dm_2\left[\frac{fa}{1-fa}\right]^{1/m_2}} + \frac{Dm_{12}^2\left[\frac{R}{(R+1)^2}\right]\left[\frac{fa}{1-fa}\right]^{2/m_{12}}}{Dm_1Dm_2\left[\frac{fa}{1-fa}\right]^{1/m_1}\left[\frac{fa}{1-fa}\right]^{1/m_2}} \quad [26]$$

Curve B in figure B2 was generated with Eq. B5, but with the correct values for Dm_{12} and m_{12} as calculated from Eqs. B2 through B4. Curve B is closer to the target, $CI = 1$ line, but there remains a problem.

B. Incorrect Combination Index Calculations for the Mutually Nonexclusive Case

Eq. 26, that suggested by Chou and Talalay (1984) and incorporated into the commercial software (Chou and Chou, 1987), is slightly wrong. This is shown by the difference between curve B in figure B2 and the $CI = 1$ line. By using a rational trial-and-error strategy, we discovered the correct form of the CI vs. fa equation for the mutually nonexclusive case for the restricted case of $m = m_1 = m_2$, Eq. B5 (Syracuse and Greco, 1986). An equivalent form of Eq. B5 has also been recently published by Lam et al. (1991). When Eq. B5 is used with the correct values of Dm_{12} and m_{12}, curve C results, the correct $CI = 1$ line.

$$CI = \frac{Dm_{12}\left[\frac{R}{R+1}\right]\left[\frac{fa}{1-fa}\right]^{1/m_{12}}}{Dm_1\left[\frac{fa}{1-fa}\right]^{1/m_1}} + \frac{Dm_{12}\left[\frac{1}{R+1}\right]\left[\frac{fa}{1-fa}\right]^{1/m_{12}}}{Dm_2\left[\frac{fa}{1-fa}\right]^{1/m_2}} + \frac{Dm_{12}^2\left[\frac{R}{(R+1)^2}\right]\left[\frac{fa}{1-fa}\right]^{2/m_{12}}}{Dm_1Dm_2\left[\frac{fa}{1-fa}\right]^{1/m}} \quad [B5]$$

C. Nonlinear Nature of the Median Effect Plot for Mutual Exclusivity with Interaction

Because of the many problems inherent with assuming a mutually nonexclusive model, one might prefer to assume a mutually exclusive model for all experimental data, including cases in which a median effect analysis shows that $m_1 = m_2 \neq m_{12}$. Combination plots generated with Eq. 25, that for mutual exclusivity (Chou and Talalay, 1984), are presented in figure B3.

$$CI = \frac{Dm_{12}\left[\frac{R}{R+1}\right]\left[\frac{fa}{1-fa}\right]^{1/m_{12}}}{Dm_1\left[\frac{fa}{1-fa}\right]^{1/m_1}} + \frac{Dm_{12}\left[\frac{1}{R+1}\right]\left[\frac{fa}{1-fa}\right]^{1/m_{12}}}{Dm_2\left[\frac{fa}{1-fa}\right]^{1/m_2}} \quad [25]$$

Curve A is the CI calculated exactly as suggested by Chou and Talalay (1984), and is the result that one would find using the commercial software (Chou and Chou, 1987) with the data in table B1. To generate curve A, Eq. 25 was used with the six parameter estimates derived from the three median effect lines of figure B1. As with the mutually nonexclusive assumption, the mutually exclusive assumption still shows an initial incorrect antagonism because of the incorrect linear extrapolation of the inherently nonlinear median effect curve for the drug combination. Curve B was also generated with Eq. 25, but with the correct values for Dm_{12} (= 4.56) and m_{12} (1.04 to 1.49). Curve B does portray the correct situation; i.e., synergism along the entire range of *fa* (with reference to the mutually exclusive model). However, because the method of Chou and Talalay (1984) does not include a reliable method to estimate Dm_{12} and m_{12} from the inherently nonlinear median effect plot for drug combinations, a useful *CI* vs. *fa* plot, such as curve B, is not readily generated.

The eight open points in figure B3 (and in figure B2) are the eight combination data points from table B1, directly plotted without the estimation of Dm_{12} and m_{12}. Instead, the raw data were plugged into Eq. 27, which depends only on the individual drug parameters, Dm_1, m_1, Dm_2, and m_2, to calculate *CI*. This approach has been discussed (Chou, 1991a), but to the best of our knowledge, is not as yet available in the commercial software (as of January, 1994).

$$CI = \frac{D_1}{Dm_1\left[\frac{fa}{1-fa}\right]^{1/m_1}} + \frac{D_2}{Dm_2\left[\frac{fa}{1-fa}\right]^{1/m_2}} \qquad [27]$$

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