AN ABSTRACT OF THE THESIS OF

DENNIS WAYNE SHELTON for the degree of MASTER OF SCIENCE
in Pharmacy (Toxicology) presented on December 14, 1979

Title: QUANTIFICATION OF THE JOINT EFFECTS OF MIXTURES OF HEPATOTOXIC AGENTS: EVALUATION OF A THEORETICAL MODEL IN MICE

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Lavern J. Weber

An approach previously developed for studying the effects of toxic mixtures on whole organism performances (ie. growth, mortality) was evaluated to determine its applicability and limitations at the organ system level. The approach was tested by quantifying the hepatotoxic effects of carbon tetrachloride (CCl₄), monochlorobenzene (MCB), acetaminophen (ACET) and selected mixtures of these compounds in male albino CF-1 mice. A quantal response was defined as an elevation in plasma alanine aminotransferase (ALT) activity > three standard deviations above the mean of pooled control values.

To determine the optimum time at which to measure the response following intraperitoneal injection, the temporal effects of each compound on plasma ALT activity were initially studied. Dose response curves were then developed for each of the hepatotoxicants administered singly and the slopes of the curves for MCB and ACET were each compared to that for CCl₄ (t test). In both cases the curves did not deviate significantly from parallelism. As a consequence, concentration addition (ie. Finney's simple
similar action) was predicted for the mixtures of both CCl$_4$+MCB and CCl$_4$+ACET. Theoretical dose response curves for the two mixtures were developed using Finney's (1971) equation describing concentration addition. The actual dose response relationship for each mixture was then empirically determined and compared to the predicted curves (χ$^2$ test). In the case of the CCl$_4$+MCB mixture, the observed dose response curve did not deviate from that predicted on the basis of concentration addition (p > 0.975). However, a similar comparison for the CCl$_4$+ACET mixture revealed a statistical difference between the observed and predicted curves (p < 0.0005). The joint effects for the mixture CCl$_4$+MCB is thus classified as concentration additive and for CCl$_4$+ACET is designated as infra-concentration additive.

The model proves to be adequate in predicting, classifying and describing the joint effects of these hepatotoxictants.
Quantification of the Joint Effects of Mixtures of Hepatotoxic Agents: Evaluation of a Theoretical Model in Mice

by

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I wish to extend my appreciation to my wife, Brenda, for her encouragement and patience. Finally, I wish to express my gratitude to my parents, Jim and Yola, for their eternal love and inspiration.
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QUANTIFICATION OF THE JOINT EFFECTS OF MIXTURES OF HEPATOTOXIC AGENTS: EVALUATION OF A THEORETICAL MODEL IN MICE

FORWARD: OVERVIEW OF THE APPROACH

Background

The concept of the dose response relationship is one of the most fundamental in toxicology. Our present understanding of this relationship is based largely upon the early work of Trevan (1927) and Gaddum (1933), who developed statistical and graphical methods to describe toxicant action. These methods, together with the development of new quantitative methods for measuring toxicity, have allowed new potentialities in comparing and describing toxicant action.

The next step in a complete understanding of the mode of action of toxicants requires an investigation of the theoretical basis of joint toxicant action. The first systematic discussion of this topic was by Bliss (1939). He recognized three principle types of joint action:

1. Independent joint action. The poisons or drugs act independently and have different modes of toxic action. The susceptibility to one component may or may not be correlated with the susceptibility to the other. The toxicity of the mixture can be predicted from the dosage-mortality curve for each constituent alone and the correlation in susceptibility to the two poisons; the observed toxicity can be computed on this basis whatever the relative proportions of the components.

2. Similar joint action. The poisons or drugs produce similar but independent effects, so that one component can be substituted at a constant proportion for the other; variations in individual susceptibility to the two components are completely correlated or parallel. The toxicity of a mixture is predictable directly from that of the constituents if their relative proportions are known.
(3) Synergistic (or antagonistic) action. The effectiveness of the mixture cannot be assessed from that of the individual ingredients but depends upon a knowledge of their combined toxicity when used in different proportions. One component synergizes or antagonizes the other.

Bliss must be noted for two important contributions from his classification scheme: 1) The toxicity of a mixture, if acting independently or similarly, can be predicted from the relative toxicities of the separate constituents applied singly, and 2) the degree of correlation between the individual susceptibilities (or tolerances) to the two components in a mixture can influence the joint action encountered.

Bliss' mathematical approach was modified by Finney (1942) in an endeavor to develop a logical relationship between the mathematical expressions for the different types of joint action. Plackett and Hewlett (1948, 1952) and Hewlett and Plackett (1952, 1959) provided a further revision of Bliss' model to a less restrictive form. These authors proposed a two-way classification scheme resulting in four distinct types of joint action, as shown in the following diagram:

<table>
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<th></th>
<th>Similar</th>
<th>Dissimilar</th>
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<tr>
<td>Non-interactive</td>
<td>Simple similar</td>
<td>Independent</td>
</tr>
<tr>
<td>Interactive</td>
<td>Complex similar</td>
<td>Dependent</td>
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Toxicant mixtures were classified as "similar" or "dissimilar" according to whether the individual toxicants acted upon the same or different sites of primary action in the organism, and as "interactive" or "non-interactive" according to whether or not one toxicant influenced the biological action.
of the other. Dissimilar joint action might also result from toxic actions that are separate in time rather than separate biochemically (Hewlett and Plackett, 1959). "Simple similar" and "independent" joint actions were considered as extreme cases in a continuum of biological possibilities. Mathematical descriptions of these expressions are precise and allow prediction of expected joint effects to serve as points of reference whereby empirical joint action can be described. The mathematical models for interactive action (i.e., complex similar and dependent) are more complex and usually require knowledge of factors unattainable by examining the effects of the single constituents and, therefore, will not be considered here.

In an effort to describe how drugs or toxicants act in a mixture, Hewlett and Plackett initially defined the hypothetical conditions of quantal response from a single drug in a single organism. After a drug is administered, only a portion of it reaches its site of action and, if the concentration is large enough, produces a quantal response. A mathematical expression depicting the relationship between the amount of drug acting, \( \omega \), and the amount of drug administered, \( z \), was given as:

\[
\omega = \mu z^n \quad (\mu, \eta > 0)
\]

The parameters \( \mu \) and \( \eta \) are values particular to the drug administered and which may vary from one individual to another in a population. Usually \( \eta = 1 \), so that \( \omega \) can often be taken as directly proportional to \( z \). That two drugs may have different values of \( \eta \) allows different dose response slopes, whether the mechanisms of action for the two drugs are the same or not.

The authors define \( \delta \) as the action tolerance of the drug. This is the concentration of drug which, after reaching the site of action, is just
sufficient to produce a quantal response. When two independently acting drugs with separate action tolerances ($\bar{\omega}_1$ and $\bar{\omega}_2$) are administered concomitantly, the condition for a non-response will be:

\[
\frac{\omega_1}{\bar{\omega}_1} \leq 1, \quad \frac{\omega_2}{\bar{\omega}_2} \leq 1
\]

(2)

Alternatively, if two drugs in a mixture act similarly, the condition for a non-response is given as:

\[
\frac{\omega_1}{\bar{\omega}} + \frac{\omega_2}{\bar{\omega}} \leq 1
\]

(3)

The intrinsic differences between independent and simple similar joint actions become implicit from (2) and (3). In the case of independent action, the response to a binary mixture is additive only if the concentrations of both drugs are above their respective threshold tolerances. The joint response can be predicted by summating the responses of the two drugs applied singly. With simple similar action, neither drug need surpass its respective threshold tolerance. Rather, if the concentrations of the respective constituents are summated (after correcting for differences in potency) and the total dose exceeds the threshold tolerance, an additive response will result. Therefore, the joint response can be predicted from the concentrations of the single components.

Anderson and Weber (1977), recognizing these differences, introduced the terms "response addition" and "concentration addition" to describe independent and simple similar joint actions, respectively. Although the mathematical descriptions of these terms are identical to their earlier
counterparts, the newer terms are less ambiguous and do not imply knowledge of mechanisms or sites of toxicant action.

Concentration Addition

When an organism is given two chemicals separately, a tolerance to each chemical is exhibited. Tolerance here refers to the maximum amount of a drug or toxicant that an animal can endure without exhibiting a quantal response; it is often called a "threshold." When a binary mixture is applied to an organism, a bivariate normal distribution of tolerances is assumed. The tolerance to one chemical may or may not be correlated with the tolerance to the other. Two drugs or toxicants acting in a concentration additive manner usually have similar enough pharmacokinetic properties and mechanisms of action, so that the correlation between tolerances to the two chemicals can be expected to be completely positive, or nearly so. The parameter \( n \) for one compound usually equals that for the other. As a consequence, the dose response curves are likely to be parallel. Exceptions to this generalization have been reported, in which similarly acting insecticides produce non-parallel dose response curves (Hewlett and Plackett, 1952). Thus, parallelism and hence complete correlation of individual susceptibilities is not a prerequisite for concentration addition, but is often an adequate assumption.

In cases where the dose response curves for the single toxicants in a mixture are parallel or when the toxic mechanisms are known to be similar, a dose response curve for their mixture can be predicted based upon the assumption of concentration addition. With the regression equations for the individual toxicants in the form of \( Y = a + b \log X \) (where \( Y \) is
the probit response to each toxicant and X is its concentration), the regression equation for a binary mixture acting via concentration addition is represented by the following equation (Finney, 1971):

\[ Y_m = a_1 + b \log(\pi_1 + p\pi_2) + \log X \]  

where,

- \( Y_m \) = probit response to the mixture
- \( a_1 \) = y intercept of the first toxicant
- \( b \) = common slope
- \( \pi_1 \) = proportion of the first toxicant in the mixture
- \( \pi_2 \) = proportion of the second toxicant in the mixture
- \( p \) = potency of the second toxicant relative to the first
- \( X \) = concentration of the mixture

**Response Addition**

Two chemicals acting by different toxic mechanisms to produce identical responses will act in such a manner that the individual responses, not the doses, are additive. A quantal response will result when the action tolerance of either one or both toxicants is surpassed. The number of individuals responding to both toxicants (i.e., both toxicants surpass their respective action tolerance thresholds) will depend upon the degree of correlation between tolerances to the two chemicals. Plackett and Hewlett (1948), recognizing this, developed predictive equations for cases of complete positive, zero and complete negative correlation of tolerances.

When the correlation of tolerances is completely positive (\( r = +1 \)), individuals most susceptible to one toxicant (A) in a binary mixture are
simultaneously most susceptible to the other (B). The proportion of individuals responding to the mixture ($P_m$) is equal to the proportion responding to the most potent toxicant in the mixture. Mathematically this is represented by:

$$P_m = P_a \text{ if } P_a \geq P_b$$

$$P_m = P_b \text{ if } P_b \geq P_a$$

(5)

where $P_a$ and $P_b$ are the proportions of individuals responding to toxicants A and B when applied singly. Cases of positive correlation of tolerances to independently acting toxicants can occur if the tolerances depend mainly upon the capacity of non-specific "sites of loss" (i.e., tissue storage, excretion) rather than upon the action tolerances.

If no correlation exists between tolerances ($r = 0$), the predictive equation is represented by:

$$P_m = P_a + P_b - P_aP_b$$

(6)

Each toxicant in this mixture produces its effect without regard to that of the other. The proportion of individuals responding to both toxicants simultaneously is determined by random probability of overlap.

The case of complete negative correlation of tolerance ($r = -1$) can occur when the same enzyme system promotes the metabolism of one toxicant to a more active form and another to a less active form. Assuming that the tolerance for both chemicals depends predominantly upon the enzyme system concerned, the most tolerant individual to one compound will be the most susceptible to the other. When less than 100% of the
individuals in a sample population are responding, no individual will respond to both chemicals simultaneously. This form of response additivity is represented by:

\[ P_m = P_a + P_b \]  (7)

A prediction based upon the assumption of response addition can be made when the dose response curves for the single constituents in a mixture are non-parallel, or when their sites of action are known to be different. Generally, the dose response curve for the mixture will be non-linear and the combination will usually be less potent than if the mixture acted via concentration addition (Finney, 1971).

In cases where an observed joint effect is greater or less than that predicted on the basis of concentration or response addition, the terms supra- and infra-additivity are designated.

The predictive equations presented here were developed and used by early authors mainly to test the effectiveness of insecticides in combination. The approach has since been extended to other toxicological problems. Anderson and Weber (1977) were able to predict, in most cases, the effects of mixtures of selected environmental toxicants on survival in guppies \textit{(Poecilia reticulata)}. Muska and Weber (1977) and Koikemeister and Weber (1979) tested the applicability of the approach using a sublethal, graded response (growth) as an indicator of toxicity in guppies. The next step in a fuller understanding of joint action is to evaluate the effects of mixtures on the organ system level. The aim of this investigation was to compare the toxic response curves of selected hepatotoxicants as a means of predicting the hepatotoxic effects of their mixtures. It is hoped that by
approaching the biochemical mechanisms of toxicant action, the underlying modes of joint action will become more evident.
REFERENCES


INTRODUCTION

With the growing concern over the hazardous wastes that are released into our environment, there is increasing interest in developing methodologies whereby threshold limits of toxicity to plants and animals can be assessed. Most existing methods are directed towards empirically determining the hazardous effects of discrete toxicants. However, environmental exposure to mixtures is far more common than exposure to single toxicants. Therefore, methods must be developed that are directed towards investigating toxic mixtures.

Considering the number of new chemicals being manufactured each year, it would be impractical to experimentally test each as a mixture and in all combinations. A sensible alternative is to develop theoretical models for predicting the joint effects of environmental toxicants. The foundations of such an approach are based upon a systematic discussion of the topic by Bliss (1939) who distinguished between types of joint action on the theoretical level, particularly for quantal type responses. Subsequently, his approach was revised by Finney (1942) and again modified by Hewlett and Plackett (1959). The model developed has been used by these authors mainly to predict insecticide effectiveness in mixtures. Anderson and Weber (1977) and Muska and Weber (1977) have extended the use of the model in predicting quantal and graded whole organism responses (i.e., mortality, growth) using aquatic organisms. The terms "concentration addition" and "response addition" were introduced in these studies to describe whether a combined effect was a function either of the concentrations
of the individual toxicants in a mixture or of the responses resulting from them, respectively (see FORWARD).

The objective in the present study was to further test the usefulness of this approach and determine its applicability and limitations on the organ system level using a mammalian species (mice). Liver damage was the specific organ system response chosen. Plasma alanine aminotransferase (ALT) activity has been shown to be a sensitive indicator of liver damage in mice (Klaassen and Plaa, 1966) and elevations correlate well with severity of damage (Belazs et al., 1961). This was the indicator selected for this investigation.

It has been noted that the type of joint action observed for a binary mixture can be influenced by the degree of separation in the durations of onset of toxic action for the respective toxicants in that mixture (Turner and Bliss, 1953). For this reason, the temporal effects of the selected hepatotoxicants used in this study on plasma ALT were examined.

The nature of the theoretical approach under investigation is such that the prediction of the joint action for a toxic mixture be performed by first investigating the separate toxicities of the components in the mixture. Therefore, preliminary single component dose response relationships were studied prior to investigation of the combined effects of selected mixtures.

It is hoped that studies such as these will have value in developing new approaches in studying hepatotoxicant mixtures as well as having implications relative to the mechanisms of action of the particular hepatotoxicants studied.
MATERIALS AND METHODS

All experiments were performed using male albino mice of the CF-1 strain, reared in our own breeding colony and housed at five per cage. The animals weighed 25-35 grams and were maintained on laboratory pellet diet (Wayne Laboratories) and water ad libitum. The animal room was maintained at a 12 hour light/dark cycle with an ambient temperature of 70-72° F.

The toxicants were carbon tetrachloride (CCl₄; reagent grade, J.T. Baker Co.), monochlorobenzene (MCB; reagent grade, Matheson Coleman and Bell, Inc.), and acetaminophen (ACET; USP grade, S.B. Penick & Co.). The CCl₄ and MCB were dissolved in corn oil and ACET was dissolved in 0.9% NaCl at 40°C. The toxicants were diluted to deliver the proper dosage in a final volume of 0.01 ml per gram of animal weight. These compounds were administered intraperitoneally between 11 A.M. and 1 P.M. each day.

Liver damage was assessed by measuring plasma alanine aminotransferase (ALT; formerly GPT) activity. Relative plasma ALT elevations were determined at 2, 4, 8, 16, 24, 48 and 72 hours following the administration of each toxicant. An optimum time interval was determined and used in the toxicant mixture study. The mice were lightly anesthetized with ether at the appropriate time and blood samples for plasma enzyme assay were drawn with a syringe rinsed in heparin. The plasma ALT determination of Reitman and Frankel (1957) was used and the results are reported as International Units (IU) per liter.

For the toxicant mixture studies, elevations in plasma ALT in
response to the hepatotoxicants were converted over to a more convenient quantal form. Any plasma ALT activity which was \( \geq 3 \) standard deviations above the mean of 56 untreated mice from our colony, was considered to indicate liver damage and a positive response.

Dose response curves describing the relationship between the dose of each hepatotoxicant applied singly and the percent of animals (plotted as probit) responding with significant liver damage, were experimentally developed for \( \text{CCl}_4 \), MCB and ACET. For each toxicant a minimum of ten animals were used at each dosage level and at least four dosage levels were administered. Certain statistical parameters from these dose response relationships were used to develop an equation predicting the dose response relationships for the joint effects of selected binary mixtures (\( \text{CCl}_4 \)+MCB and \( \text{CCl}_4 \)+ACET). Either concentration or response addition were predicted, depending upon which appeared more probable. If the slopes for the single component curves for two toxicants are found to be statistically different (t test; Dixon and Massey, 1969), response addition would be the most probable type of joint action encountered for the mixture and thus, would be predicted. If the curves can not be shown to deviate significantly from parallelism, concentration addition would be predicted. The predictive equations are derived from Finney's (1971) simple similar joint action (concentration addition) and Plackett and Hewlett's (1948) independent action (response addition).

Dose response curves for the mixtures \( \text{CCl}_4 \)+MCB and \( \text{CCl}_4 \)+ACET were experimentally developed. The total dose of the mixture was increased while maintaining a constant dose ratio between the toxicants (\( \mu \text{mole/kg basis} \)). The hypothesis that the observed points did not differ
significantly from the corresponding predicted coordinates was tested (Chi square test, Finney, 1971). If this hypothesis could not be disproven, the observed joint effect would be categorized as either response or concentration addition, depending upon which type had been predicted. If the observed and predicted curves differed significantly, then either infra- or supra-addition would be used to describe the effects of the mixture.
RESULTS

Preliminary Studies

Figure 1 shows the relative plasma ALT activity plotted against time following administration of CCl₄, MCB or ACET. While the response to CCl₄ maximized at 16 hours and was beginning to wane, the elevations from MCB and ACET continued until they reached a maxima at 24 hours. Thus, within 24 hours after injection, the hepatotoxic effects of each hepatotoxicant had become evident. Therefore, for the toxicant interaction studies, blood was obtained from each mouse 24 hours after i.p. injection of the single toxicants or binary mixtures. The mean plasma ALT activity from 56 control mice was determined to be 10 ± 2 (S.D.) I.U. The cut-off point for a quantal response was thus set to be 16 I.U.

Single component dose response curves were initially developed for each hepatotoxicant. Characteristics derived from these curves (ie. slope, potency ratio, TD50) are shown in Table 1. The TD50's were used to calculate the potency ratios for the toxicants. MCB and ACET were found to be approximately equipotent in producing liver damage whereas CCl₄ appeared about 35 times more toxic than either MCB or ACET. Consequently, we decided to test the joint hepatotoxic effects of the mixtures CCl₄+MCB and CCl₄+ACET. The large potency ratio between the constituents in the tested mixtures allowed greater resolution in differentiating the possible types of joint action resulting from them. When the slopes of the MCB and ACET curves were each compared to that of CCl₄ (t test), no significant deviation from parallelism was apparent (Table 1).
Figure 1. Temporal pattern of plasma alanine aminotransferase activity in male albino mice following intraperitoneal injection of carbon tetrachloride (CCl₄, 20.7 mg/kg), monochlorobenzene (MCB, 553 mg/kg) and acetaminophen (ACET, 630 mg/kg). Each bar represents the mean (± SEM) of five animals and the control is a mean of 56 animals. Approximately equitoxic dosages were used.
Table 1. Dose Response Characteristics of Selected Hepatotoxicants on Plasma Alanine Aminotransferase Activity\textsuperscript{a} in Male Albino Mice.

<table>
<thead>
<tr>
<th>Toxicant</th>
<th>TD50\textsuperscript{b} mg/kg</th>
<th>TD50\textsuperscript{b} µmole/kg</th>
<th>Potency Ratio\textsuperscript{c}</th>
<th>Slope (±S.D.)</th>
<th>T Value\textsuperscript{d}</th>
<th>p Value\textsuperscript{e}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Carbon tetrachloride (CCl\textsubscript{4})</td>
<td>16.9 (14.2-19.9)</td>
<td>109.5 (92.5-129.6)</td>
<td>1.0</td>
<td>6.57 (± 1.19)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>2. Monochlorobenzene (MCB)</td>
<td>428 (395-466)</td>
<td>3807 (3505-4136)</td>
<td>34.8</td>
<td>8.40 (±1.24)</td>
<td>-.45</td>
<td>0.667</td>
</tr>
<tr>
<td>3. Acetaminophen (ACET)</td>
<td>558 (485-643)</td>
<td>3694 (3209-4252)</td>
<td>33.7</td>
<td>8.31 (± 1.15)</td>
<td>-.77</td>
<td>0.471</td>
</tr>
</tbody>
</table>

\textsuperscript{a} A positive response is defined as a plasma alanine aminotransferase elevation > 3 standard deviations above the control mean (10 ± 2 I.U.).

\textsuperscript{b} Determined from the dose response regression equation. Brackets indicate 95% C.I.

\textsuperscript{c} TD50 (MCB or ACET)/TD50 (CCl\textsubscript{4}); µmole/kg comparison.

\textsuperscript{d} T value determined when slope of dose response curve for MCB or ACET is compared to that of carbon tetrachloride.

\textsuperscript{e} In each case slopes were not significantly different from parallel at the p value indicated.
It was evident from these findings that concentration addition would be the most likely effect for the mixtures CCl$_4$+MCB and CCl$_4$+ACET and therefore was predicted in each case.

**CCl$_4$+MCB Mixture**

A theoretical dose response curve for the binary mixture of CCl$_4$ and MCB at a molar dose ratio of 1:38 was predicted using Finney's (1971) equation for concentration addition. The development of this curve involved utilization of data from the single component dose response regression equations as well as a common regression coefficient determined by analysis of covariance. This curve is shown plotted in Figure 2 along with the curves for CCl$_4$, MCB and the observed curve for the 1:38 mixture. Regression equations describing these curves are given in Table 2, which also summarizes the results of the Chi square test comparing the predicted with the observed dose response curve. The results show no difference between the two curves at $p > 0.975$.

**CCl$_4$+ACET Mixture**

The predictive equation for the mixture of CCl$_4$+ACET was developed for a molar dose ratio of 1:36.6 (CCl$_4$:ACET). This curve is shown plotted in Figure 3 along with the observed dose response curve for the mixture as well as those for the singly applied CCl$_4$ and ACET. The regression equations describing these four curves are given in Table 2. The test of comparison revealed that the predicted and observed curves for the CCl$_4$+ACET mixture differ ($p < 0.0005$). The observed joint effect
Figure 2. Dose response curves illustrating the effects of carbon tetrachloride (CCl₄), monochlorobenzene (MCB) and the 1:38 mixture (CCl₄:MCB) on the percent of animals (expressed as probit) responding with significant plasma alanine aminotransferase elevations. Both the predicted and the observed curves for the mixture are shown. Each point represents a treatment of a minimum of ten animals.
Table 2. Regression Equations Describing the Dose Response Curves for Carbon Tetrachloride (CCl₄), Monochlorobenzene (MCB), Acetaminophen (ACET) and Selected Mixtures. The number of animals responding with significant liver damage (Y) is expressed as a function of toxicant dose (X).

<table>
<thead>
<tr>
<th>Toxicant</th>
<th>Regression Equation b</th>
<th>Correlation Coefficient</th>
<th>$\chi^2$</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. CCl₄</td>
<td>$Y = -8.40 + 6.57 \log X$</td>
<td>0.940</td>
<td>----</td>
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<tr>
<td>2. MCB</td>
<td>$Y = -25.07 + 8.40 \log X$</td>
<td>0.979</td>
<td>----</td>
<td>----</td>
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<tr>
<td>3. ACET</td>
<td>$Y = -24.66 + 8.31 \log X$</td>
<td>0.980</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>4. CCl₄+MCB (1:38)</td>
<td>Observed: $Y = -16.86 + 6.70 \log X$</td>
<td>0.957</td>
<td>0.998</td>
<td>$&gt;0.975^c$</td>
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<tr>
<td></td>
<td>Predicted: $Y = -16.89 + 6.68 \log X$</td>
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<tr>
<td>5. CCl₄+ACET (1:36.6)</td>
<td>Observed: $Y = -26.12 + 8.89 \log X$</td>
<td>0.868</td>
<td>40.5</td>
<td>$&lt;0.0005^d$</td>
</tr>
<tr>
<td></td>
<td>Predicted: $Y = -17.11 + 6.93 \log X$</td>
<td></td>
<td></td>
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</tbody>
</table>

*a* Significant liver damage is defined as an elevated plasma alanine aminotransferase $>$ 3 standard deviations above the control mean.

*b* Y is expressed as probits and X as $\mu$moles per kilogram.

*c* Observed and predicted dose response curves for the CCl₄+MCB mixture were shown not to be significantly different at the p level indicated.

*d* Observed and predicted dose response curves for the CCl₄+ACET mixture were shown to be significantly different at the p level indicated.
Figure 3. Dose response curves illustrating the effects of carbon tetrachloride (CCl₄), acetaminophen (ACET) and the 1:36.6 mixture (CCl₄:ACET) on the percent of animals (expressed as probit) responding with significant plasma alanine aminotransferase elevations. Both the predicted and the observed curves for the mixture are shown. Each point represents a treatment of a minimum of ten animals.
for the mixture can thus be categorized as infra-additive on the basis of concentration addition.

To determine if response additivity might more adequately describe the observed joint effect for the CCl₄+ACET mixture, the observed points were statistically compared to those predicted on the basis of Plackett and Hewlett's (1948) independent action (response additivity). The findings show that the observed and predicted curves again differ ($\chi^2(5) = 40.6; p < 0.0005$).
DISCUSSION

Many of the safety limitations determined for environmental toxicants are derived from studies of their effects on whole organism performances, such as mortality. However, most toxic manifestations from environmental pollutants are exhibited as sublethal responses. It is therefore of value to investigate the effects of toxic mixtures on a sublethal level. Because liver toxicity can easily be quantified, this organ serves as a good experimental model whereby a more sensitive evaluation of sublethal, joint toxic effects can be performed. Bioactivation in the liver accounts for the relatively selective hepatotoxicity of many foreign compounds. It has been indicated that activation plays a major role in CCl₄ (Slater, 1966), MCB (Brodie, et al., 1971) and ACET (Mitchell et al., 1973) induced hepatotoxicity. The toxicity of all of these compounds is manifested histologically as centrolobular necrosis (Gillette, 1972). A major contributor to the toxic mechanism of each of these chemicals is thought to be covalent binding of the metabolite to intracellular macromolecules. It is possible that this similarity in toxic action is responsible for the parallel dose response curves observed in this study.

The results of the present investigation suggest that the toxicity of hepatotoxic mixtures can be predicted and classified by examining the single constituent toxicities. The joint effects observed for the CCl₄+MCB mixture were clearly predicted by the equation for concentration addition. It is evident then, that the hepatotoxic response of a given dose of a CCl₄+MCB mixture is not merely the sum of the toxic effects of the CCl₄ and MCB given singly. Instead, the addition of the effects follows a
log-linear relationship with respect to the total concentration of both CCl$_4$ and MCB in the mixture. This could obviously have practical implications since a toxic mixture acting via concentration addition is usually more toxic than if the same two toxicants act via response addition (Finney, 1971).

The interpretation of the joint effects of the CCl$_4$+ACET mixture is more difficult. There is an apparent antagonism exhibited with a resultant infra-additivity. Since present knowledge of the toxic mechanisms for both MCB and ACET does not present any striking differences between the two, any observed differences in joint action, when combined with CCl$_4$, is largely unexplained. It has been inferred that acetaminophen may damage the hepatic endoplasmic reticulum (Thorgeirsson, 1973). If this is the case, then this could affect the activation of CCl$_4$, with resulting infra-additivity.

Plackett and Hewlett (1952) classified joint effects into two broad categories: non-interactive and interactive. The predictive equations in this study are based upon the assumption that each toxicant in a mixture does not influence the amount of the other reaching the site of action or the changes elicited by the other at the site of action (ie. non-interactive). It is possible that this assumption does not apply to the case of the CCl$_4$+ACET mixture. The approach used in this study has thus proven to be of practical value in raising questions on the mechanistic level.

Besides being of practical value, the present approach, utilized on the organ system level, can also have theoretical implications. For instance, in the case of simple similar action (ie. concentration addition), as originally proposed by Bliss (1939) and Finney (1942), there is a
limiting requirement that the dose response curves for the individual toxicants in a mixture be parallel. Because of this assumption, some workers find their approach untenable (Plackett and Hewlett, 1952; Ball, 1959; Dunnett, 1968). Hewlett and Plackett (1959) have modified the approach to apply whether the slopes are equal or not. A non-parallelism occurs when differences in biological variation of tolerances to the similarly acting toxicants occurs, which is not necessarily a tolerance at the site of toxic action. The question can be asked then; as one approaches the site of toxic action, at what point, if at all, does parallelism between the dose response curves of the single toxicants in a mixture become obligatory if the respective mechanisms of action are the same? This question can be answered by studying biochemical mechanisms of toxicity within organ systems.

In summary, the mathematical approach has proven valuable in predicting and describing the joint effects of a mixture of CCl₄ and MCB as concentration additive. In the case of the mixture of CCl₄ and ACET, the usefulness of the model was demonstrated by allowing prediction of an expected response (concentration addition) to serve as a frame of reference whereby the observed joint action could be categorized. Since the observed and predicted joint effects for the CCl₄+ACET mixture differed, questions are raised as to the nature of the interaction on the mechanistic level.
REFERENCES


In Table 3, a comparison is made between the experimentally observed responses resulting from the 1:38 CCl$_4$+MCB mixture and the expected responses, predicted on the basis of both concentration and response addition. Selected dosages of CCl$_4$ and MCB were summated at a constant ratio and the total dosage of the mixture (as µmole/kg) was determined. When these combined dosages were fitted into the predictive equation calculated on the basis of concentration addition (Table 2), expected responses for this type of addition were computed.

The joint effects predicted on the basis of response addition were calculated by fitting the expected single constituent responses (calculated from single component regression equations) into Plackett and Hewlett's (1948) equation for independent action, with zero correlation of tolerances.

It is evident that the predicted outcome for concentration additivity is of greater magnitude than that for response additivity. The observed effects for the CCl$_4$+MCB mixture do not significantly differ from those predicted on the basis of concentration addition.

Table 4 shows a similar comparison for the 1:36.6 CCl$_4$+ACET mixture. The observed joint effects differ from the responses predicted on the basis of both concentration and response addition. Since the observed dose response curve for this mixture (Figure 3) appears to be a straight line, parallel to the single component curves, the joint action is probably infra-concentration additive.
<table>
<thead>
<tr>
<th>Toxicant</th>
<th>Non-Combined Dose (µmole/kg)</th>
<th>Combined Dose (µmole/kg)</th>
<th>Predicted Non-Combined Response (%)</th>
<th>Predicted Combined Response (%)</th>
<th>Observed Combined Response (%)</th>
<th>Sample Size</th>
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</table>

\(^a\) R. A. = response addition as predicted using Hewlett and Plackett's (1948) equation for independent joint action.

C. A. = concentration addition as predicted using Finney's (1971) equation for simple similar action.
Table 4. Predicted and Observed Responses: A Quantal Evaluation of Hepatotoxicity in Male Albino Mice Exposed via Intraperitoneal Injection to a Mixture of Carbon Tetrachloride (CCl₄) and Acetaminophen (ACET) at a Ratio of 1:36.6.

<table>
<thead>
<tr>
<th>Toxicant</th>
<th>Non-Combined Dose (μmole/kg)</th>
<th>Combined Dose (μmole/kg)</th>
<th>Predicted Non-Combined Response (%)</th>
<th>Predicted Combined Response (%)</th>
<th>Observed Combined Response (%)</th>
<th>Sample Size</th>
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<td></td>
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<td>70</td>
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<td>0.3</td>
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</tbody>
</table>

²R. A. = response addition as predicted using Hewlett and Plackett's (1948) equation for independent joint action.

C. A. = concentration addition as predicted using Finney's (1971) equation for simple similar action.