Symposium

Synergism, Antagonism, and Additive Action of Fungicides in Mixtures

Procedures for Calculating and Differentiating Synergism and Antagonism in Action of Fungicide Mixtures

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Fungicides are combined in mixtures for three main reasons: (i) to widen the spectrum of antifungal activity; (ii) to exploit synergistic interactions between the fungicides, by which the overall activity can be increased or the amounts used can be reduced without loss of activity; and (iii) to delay the selection of resistant strains (14). The properties (i and iii) of an arbitrary mixture can be tested by biological methods, whereas to measure synergism special mathematical processing is needed. To proof the occurrence of a synergistic effect between pesticides, biological evidence alone is not enough, because the observed efficacy of a mixture should be compared with its expected efficacy. The latter is a result of calculation according to a specified theory of nonsynergistic action.

Several methods have been developed to assess synergistic interaction between pesticides. However, only two basic approaches (sometimes modified) are commonly used: the Abbott (also known as the Colby) method (1,7), which is usually applied for mixtures whose constituents produce their effects in different modes, and the Wadley method (35,36), which is applied for mixtures with similar action of the components.

The purpose of this paper is twofold: (i) to critically review some important approaches for estimating the joint action of pesticides, and (ii) to propose a classification of pesticide interaction in a mixture with the aim of developing class-specific methods that might increase the accuracy of synergism analysis.

DEFINITIONS AND CLASSIFICATIONS

Despite the long history of the study of pesticide mixtures, clear, generally accepted definitions of synergism and antagonism are still absent. For example, according to the terminology of Gaddum (13), synergism is declared when the effect of the mixture exceeds the effect of its more potent component. However, Hewlett (19) and Gisi (14) define synergism as a joint action of mixture components in which the total effect is greater than the sum of the effects of the individual components. Gaddum's (13) definition is appropriate only in the case of potentiation, when only one component of the mixture is bioactive if applied alone. On the other hand, comparing efficacy of the mixture with the "sum of the effects" of its constituents is suitable in the case of potentiation as well, because the effect of an inert component can be considered zero. More details on the different definitions of synergism appear in Putnam and Penner (26), Banki (3), and Morse (23).

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To make the definition of synergism more accurate and less ambiguous, we use the term "expected effect of the mixture" instead of "sum of the effects of the individual components." According to Gowing (16) "the expected effect" is a simple summation of the effects of the materials acting alone, whereas according to Wadley (36) and Colby (7) it is a value that should be calculated by specific formulas.

The methods of Abbott (1,7) and Wadley (35,36) were originally developed for insecticides but were extensively studied with fungicides as well (15). Responses measured with fungicides usually include diseased leaf area in both the greenhouse and the field. Percent survival of germlings and linear growth of mycelial mats in vitro also may be used. Choosing either method depends on the possible type of action of the components in the mixture. Two types are usually considered: different and similar action (6, 14,17,22,36).

Different action occurs when each pesticide affects a different physiological activity or vital system in the pest (36). In this case, the Abbott procedure was proposed to determine synergism. Finney (10) suggested that this method can be used when the chemicals either act independently or sequentially.

Similar action occurs when the same vital systems in the pest are affected by both pesticides. When this is the case, one component of the mixture could be replaced by the other at a response-equivalent dose. The Wadley method is appropriate to calculate synergism when chemicals can be substituted for one another at a fixed ratio. Otherwise, synergism analysis becomes complex, and "it seems unlikely that synergism will be found under such conditions" (36).

An important point is whether the components affect pest development simultaneously or separately over the course of time. This may influence the estimation of the expected efficacy of the mixture. Therefore, we propose to complete the classification according to the following mechanisms of possible joint action of pesticides: different simultaneous action, similar simultaneous action, and nonsimultaneous action. In the first case, we accept an independent action of the mixture components, i.e., no dependent efficacy between components is expected when pesticides are used together. In the last two cases, the efficacy of one component depends on the efficacy of the other, i.e., the joint action is certainly dependent. Thus, the dependent action of pesticides does not generally mean synergistic or antagonistic interaction, except apparently in the case of different simultaneous action.

DETERMINATION OF SYNERGISM

We shall consider an arbitrary mixture consisting of two active ingredients. Generalization for more components is possible (7,18,

29). Determination of a synergistic (or antagonistic) property of a mixture requires the comparison of the experimental results with a reference model that represents the joint action of the mixture constituents under the assumption of the absence of either synergism or antagonism. The activity of a mixture is first measured experimentally, and then it must be compared with its calculated expected activity to achieve an indication of the extent of pesticide interaction. Thus, the decision about synergism is entirely dependent on the assessed value of the expected response of the mixture. An underestimation of the expected effect will result in an overestimation of the synergism.

We shall see later that synergism may be estimated in two ways: (i) by the "synergy ratio," which is the ratio between two efficacy levels (percent killed or percent controlled), the observed versus the expected; and (ii) by the "cotoxicity ratio," which is the ratio between two doses (milligrams per liter, etc.), the expected versus the observed, that provide the same level of control. These ratios are not directly comparable because they are inconsonant.

To reveal synergism (or antagonism), the dose-response curves or the efficacies of the mixture and its constituents usually must be obtained in separate experiments. There is one method, the calculus method of Drury (8), that needs no information about the response of the pest to the individual agents alone.

The Drury Method of Calculus

Drury (8) used calculus to characterize the interaction of two agents (herbicides). He proposed the following algorithm:

Step 1: Determine a multiple regression polynomial that describes response $f(x,y)$ to a mixture of two components in amounts x and y.

Step 2: Calculate the first partial derivatives of function $f(x,y)$ with respect to each constituent, $\partial f(x,y)/\partial x$ for the action of x and $\partial f(x,y)/\partial y$ for the action of y, and the second-order partial derivative of f with respect to x and y, $\partial^2 f(x,y)/\partial x \partial y$, for the interaction.

Step 3: Calculate numerical values of three derivatives obtained at points of interests, i.e., for specific mixtures that consist of two agents in amounts x and y.

Step 4: Compare the sign of the interaction, $\partial^2 f(x,y)/\partial x \partial y$, with the signs of the actions of each agent, $\partial f(x,y)/\partial x$ and $\partial f(x,y)/\partial y$. The type of interaction is determined by agreement or disagreement in the obtained signs. "If all three derivatives agree in sign, then x and y are both promoting the action of one another." This mutual promotion is called synergism. "If the actions of x and y agree in sign, but the interaction is of opposite sign, then x and y are reversing the action of one another.... This is mutual antagonism." If the actions have opposite signs and one (e.g., x) agrees in sign with the interaction, whereas the other (e.g., y) does not, "then y is promoting the action of x, whereas x is reversing (is antagonistic to) the action of y."

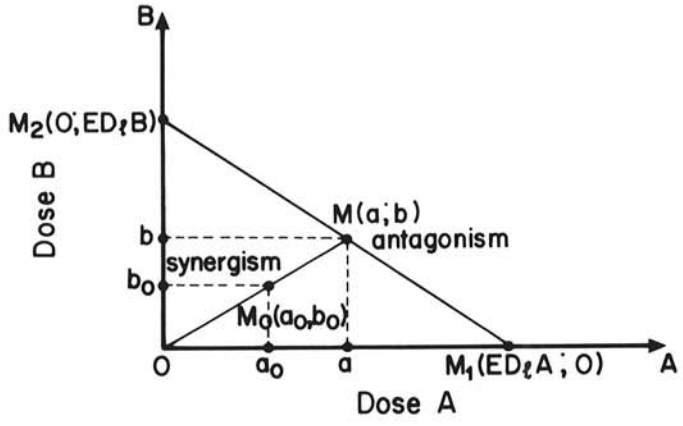

Fig. 1. Wadley's isobole for two pesticides, A and B. The intensity of synergy can be measured by the "cotoxicity ratio": $Int = OM/OM_0$.

The Drury method of calculus characterizes the interaction of two components in a mixture and shows whether it is positive or negative. The revealed interaction does not necessarily indicate synergism or antagonism. Nash (24) emphasized that the results of this calculus method could be questionable. Positive (or negative) interaction and synergism (or antagonism) may represent two different phenomena. Despite an interaction, one cannot conclude whether the response to a mixture is above or below some expected level. Nevertheless, Drury's "synergism" probably could be useful in selecting mixtures suspected to be synergistic.

Banki (3) separated all methods for measuring joint action into two groups: empirical and biometrical. We follow this classification.

Empirical Methods of Joint Action

The empirical approaches for determining synergism are divided into two categories (23): the additive-dose model (ADM) and the multiplicative-survival model (MSM). The distinguishing characteristic of the ADM is that the components of the mixture can be substituted for each other at an equivalent dose. The doses of equivalent effect for an arbitrary fixed level of response are obtained from dose-response curves of the constituents when acting alone. The Wadley (isobole) method (35,36) and the Morse additive-dose approach (23) belong to the ADM. Nash (24) also included the two-parameter method of Rummens (29,30) and the calculus method of Drury (8) in the ADM. We see in the Drury approach nothing in common with the ADM. A substitution of the mixture components is not implemented in the Rummens scheme (described below), and therefore, it is not entirely an ADM.

The Abbott method (1,7), the Gowing formula (17), and the Morse multiplicative-survival approach (23) are attributed to the MSM. These models estimate survival of a pest population after exposure to a mixture similarly. If the action of the first and the second components of the mixture results in survival of proportions q_1 and q_2 of the target population, respectively, then the expected survival resulting from application of the mixture is calculated as the product $q_1 q_2$.

The ADM and MSM correspond to cases of similar and different joint action, respectively. Therefore, information about the biological mode of action of the components in the mixture, whether they affect the same systems, is desirable. The distinction between ADM and MSM is not always recognized, which may result in misleading conclusions concerning a specific mixture.

There are two ways to assess joint action based on the reference model. In the first, called the direct method (2), the observed response (percent killed or percent disease controlled) of a specific mixture is directly compared with the expected response calculated according to the model. The "synergy ratio," R, between the observed, C_{obs}, and expected, C_{exp}, efficacies of the mixture is calculated as

$$R = C_{obs}/C_{exp} \quad (1)$$

If R is greater than, equal to, or less than 1, then the interaction between pesticides is synergistic, expected, or antagonistic, respectively. The value of R reflects the relative intensity of the joint action: the greater the R than 1, the stronger the synergism; the lesser the R than 1, the more intense the antagonism.

In the second approach, called the indirect method, the dose of the mixture expected to give a fixed response (percent killed or percent disease controlled) is predicted based on the model and is compared with the experimental mixture dose that provides the same response. For a certain mixture made up of a known proportion of two pesticides, the ratio is calculated between the expected and the actual doses that provide a fixed level of efficacy. When this "cotoxicity index" (33,34) is greater or less than 1, synergism or antagonism is indicated, respectively. This procedure is the basis of the isobole method, which is a convenient graphic

procedure for investigation of synergism. For any one reference model, the direct and isobole (indirect) methods should not result in contradictory conclusions.

The Wadley Method

The Wadley method was developed for the case of similar action of insecticides in a mixture. The possibility of replacing one pesticide with an equivalent effective dose of the other and a constant ratio between equipotential doses are assumed. The latter implies parallelism of the dose-response curves of the components after log dose-probit response transformation (12,36). When the transformed curves deviate considerably from parallel lines, the Wadley procedure may be inappropriate for determining synergism.

The Wadley method is indirect. The expected effective dose of a mixture is estimated based on the effective doses of the same level of the mixture's constituents. It is then compared with the actual dose of the mixture, which provides the same effect. When the expected dose is greater than, equal to, or less than the observed experimental dose, synergism, additivity, or antagonism, respectively, is declared.

Let us denote ED_lM, ED_lA, and ED_lB as the equally effective doses of mixture M (the expected dose) and pesticides A and B, respectively, for an arbitrary fixed level of disease control, l. If a ($a \leq ED_lA$) and b ($b \leq ED_lB$) are the absolute amounts of pesticides A and B, respectively, in mixture M that provides a given level of control, l, then $ED_lM = a + b$.

Dose b of pesticide B can be replaced by equipotential dose $ED_lA - a$ of pesticide A, so application of $a + (ED_lA - a) = ED_lA$ units of A results in an l level of disease control. The constant ratio, k, between equally effective doses of pesticides A and B is assumed. This principal assumption of the Wadley method implies the equations

$$ED_lB = kED_lA \text{ and } k = ED_lB/ED_lA$$

and

$$b = k(ED_lA - a) = (ED_lB/ED_lA)\,(ED_lA - a) = ED_lB - (aED_lB/ED_lA)$$

This results in the following identity:

$$(a/ED_lA) + (b/ED_lB) = 1 \qquad (2)$$

which holds true for any a and b. The expected effective dose of the mixture is

$$ED_lM = (a + b)/[(a/ED_lA) + (b/ED_lB)] = 1/[(p_A/ED_lA) + (p_B/ED_lB)] \qquad (3)$$

in which $p_A = a/(a + b)$ and $p_B = b/(a + b)$ are the proportions of pesticides A and B in the mixture, respectively.

Graphic representation of the Wadley scheme, proposed by Tammes (35) and considered in Hewlett (20), is called the isobole method. Let mixture M consist of amounts a and b of constituents A and B, respectively. If two perpendicular axes, A (horizontal) and B (vertical), on the Cartesian plane are considered, then matching mixture M to the point with nonnegative coordinates (a,b) results in one-to-one correspondence between all possible two-component mixtures and all points in the first quadrant (Fig. 1).

Let us consider only those mixtures ($ED_lM = a + b$) that provide an arbitrary fixed level, l, of control subject to the principal Wadley's assumption that any amount of one component can be replaced by the other in a constant proportion; if so, equality (2) is fulfilled. Geometrically, this means that corresponding points (a,b) for all considered mixtures lie on the straight line that connects two points with coordinates M_1 (ED_lA,0) and M_2 (0,ED_lB) on axes A and B, respectively (Fig. 1). This line is called the isobole. The efficacy of any other mixture (corresponding to a point lying outside the isobole constructed) differs from l. One can prove that the isoboles for different levels of mixture efficacy are parallel lines.

Thus, the isobole of fixed level l represents all mixtures expected to provide a given level of control. Let us consider an arbitrary mixture that corresponds to point M (a,b) on the isobole (Fig. 1). To determine synergism, different concentrations of the mixture with a fixed ratio, a/b, of the components must be tested, and the corresponding dose-response curve should be constructed. Dose $a_0 + b_0$ of the mixture, which demonstrates a given level of efficacy, l, is experimentally established, and point M_0 (a_0,b_0) corresponding to this observed effective dose is plotted on the plane. Because $a/b_0 = a/b$, points M, M_0, and O belong to the same straight line (Fig. 1). If "experimental" point M_0 falls between points O and M, then $a_0 < a$ and $b_0 < b$, and the mixture is synergistic for the given control level. If M_0 falls outside triangle OM_1M_2, then $a_0 > a$ and $b_0 > b$, and the mixture is antagonistic.

The intensity, *Int*, of the synergistic (or antagonistic) interaction is defined by the cotoxicity ratio between the expected, $a + b$, and the observed, $a_0 + b_0$, effective doses for a fixed level of control. This is geometrically expressed (Fig. 1) by the formula $Int = OM/OM_0$.

It is possible to determine the optimal mixing proportion to achieve the desired level of disease control with a minimum total amount of the components (isobologram [6,22]). The proportion is optimal for a mixture with the maximal intensity of synergistic interaction. If the observed effective doses of mixtures with different proportions of the components are plotted, the optimal proportion can be estimated graphically (6,22).

The isobole method provides an excellent pictorial view of the joint action of two components in a mixture and has value as a graphic tool for the study of synergism. The direct version of the Wadley approach, on the other hand, is almost ignored by researchers, although it could be useful in investigating any specific mixture with similar action of components and parallel dose-response curves after the log-probit transformation.

In the direct method, consider a mixture that consists of two components, A and B, in amounts a and b, respectively, with the constant ratio, k, between equally effective doses of A and B. If a_1 and b, b_1 and a are doses of equal efficacies, then dose b of component B in the mixture can be substituted by dose $a_1 = b/k$ of A, and vice versa, dose a of A can be replaced by $b_1 = ka$ of B. This implies that the efficacy, C_{exp}, of the mixture is expected to be equal to the efficacy, C_A, of A, which is applied alone in dose $a + a_1$, and to the efficacy, C_B, of B, which is applied alone in dose $b_1 + b$. Such an estimate of the expected efficacy of the mixture, $C_{exp}(a + b)$, is correct if $C_A(a + a_1) = C_B(b + b_1)$, which means that $a + a_1$ and $b_1 + b$ have to be equally effective doses. This condition is fulfilled because $b_1 + b = k(a + a_1)$. Hence, the expected efficacy of the mixture can be calculated by the following algorithm:

Step 1: Plot dose-response curves for each component in the same figure (Fig. 2). They should be parallel in log-probit scales.

Step 2: Point dose a and b of components A and B, respectively, on the dose scale.

Step 3: Determine the equipotential dose, a_1, of constituent A for dose b of B (Fig. 2).

Step 4: Determine the effect, $C_A(a + a_1)$, of dose $a + a_1$ of ingredient A (Fig. 2) and use this value as the estimate of the expected mixture efficacy, C_{exp}.

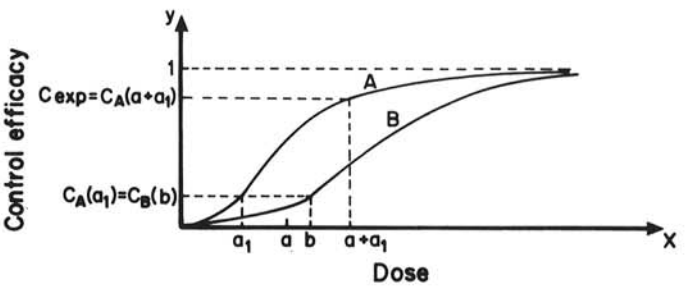

Fig. 2. The expected mixture efficacy, C_{exp}, calculated according to the "direct" version of the Wadley method.

Synergism (or antagonism) of the mixture and its intensity is estimated according to equation 1.

The hypothesis of equal slopes for dose-response curves of the components of a mixture after the log-probit transformation can be examined by means of different statistical tests (11,32). If this hypothesis is not accepted, the Wadley procedure may lead to erroneous results. For cases with nonparallel dose-response curves of mixture constituents, other procedures have been developed for calculating synergism. We will consider two of them.

The Morse ADM

The ADM of Morse (23) was designed to study synergism of an arbitrary mixture with no assumption of the form of dose-response curves of the components. Despite the fact that the Morse method provides a more accurate estimate of the expected efficacy of any mixture compared to that of Wadley, it is correct only for cases when the potency of one component of a mixture relative to the other is constant at all levels of response.

Morse (23) considered a mixture, S_m, that consists of two components, S_1 and S_2, in a constant ratio, $x_1/x_2 = \pi_1/\pi_2$, where $\pi_1 + \pi_2 = 1$, x_1 and x_2 are arbitrary doses of S_1 and S_2, respectively. The idea of the Morse method is to construct the expected dose-response curve for the mixture when the S-shaped curves of components S_1 and S_2 are known. Building the curve for mixture S_m, demonstrated in Figure 3, is done as follows:

Step 1: Plot the dose-response curves for components S_1 and S_2.

Step 2: Draw a horizontal line at any specific level of response, $Y^\star$, that cuts the curve for S_1 at C and the curve for S_2 at E. Determine Z_1 and Z_2.

Step 3: Calculate x_m according to the formula

$$x_m = (Z_1Z_2)/(\pi_2Z_1 + \pi_1Z_2) \quad (4)$$

Plot this value on the dose axis, and draw a vertical line through the point $(x_m,0)$. The intersection of the vertical and horizontal lines will be the point of the dose-response curve for mixture S_m.

Step 4: Repeat steps 2 and 3 for different levels of response, and connect the constructed points to obtain the expected dose-response curve for mixture S_m.

The direct version of the Morse ADM is realized by the synergy ratio between the expected and observed responses of x_m units of mixture S_m, which read off the expected and actual dose-response curves, respectively.

The isoboles of the Morse method are shown in Figure 4. The equation (compare with equation 2)

$$(x_1/Z_1) + (x_2/Z_2) = 1 \quad (5)$$

is fulfilled (23) for any mixture comprised of arbitrary doses, x_1 and x_2, of components S_1 and S_2, respectively, that is expected to provide a fixed constant response, $Y^\star$. This defines the isobole, which is a straight line through points $(Z_1,0)$ and $(0,Z_2)$ (Fig. 4). Values Z_1 and Z_2 are obtained from the dose-response curves for S_1 and S_2 (Fig. 3). Unlike the Wadley method (Fig. 2), the isoboles for different levels of response are not necessarily parallel. They are parallel if the ratio Z_1/Z_2 is constant for all values of response $Y^\star$. In this case, the parallel Morse isoboles are reduced to those of Wadley if the dose-response curves are described by the function of log-normal distribution (5). Determining synergistic (or antagonistic) interaction of the mixture components by means of the Morse isoboles is implemented in the same way as for Wadley's isoboles.

Morse tried to adjust Wadley's idea of replacing one component of a mixture with the other to the case of variable ratios of doses of equal potency. However, his proposal (23) to replace x_2 units of S_2 in mixture $x_m = x_1 + x_2$ by $x_2(Z_1/Z_2)$ units of S_1 means that the variable ratio $\rho = Z_1/Z_2$ between equipotential doses Z_1 and Z_2 of constituents S_1 and S_2, respectively, is extrapolated for the dose $x_2 < Z_2$ of S_2. This implies the improbable conclusion that the dose of S_1 equipotential to x_2 varies depending on the amount x_1 of S_1 in the mixture. Thus, the Morse method may be considered as a heuristic procedure for determining synergism, and it is indeed correct only when the ratio, $\rho = Z_1/Z_2$, between equipotential doses is constant, i.e., under the principal assumption of the Wadley approach.

The Rummens Two-Parameter Method

Rummens (29) described dose-response S-shaped curves by the equation

$$Y = 1 - 1/[1 + (x/c)^b] \quad (6)$$

in which Y is the proportion of the pest population controlled by dose x of a pesticide, c is the dose that kills one-half of the pest population, and b is a dimensionless parameter defining the bend in the response curve.

The procedure of Rummens enables us to construct the expected curve of a mixture on the basis of the experimental dose-

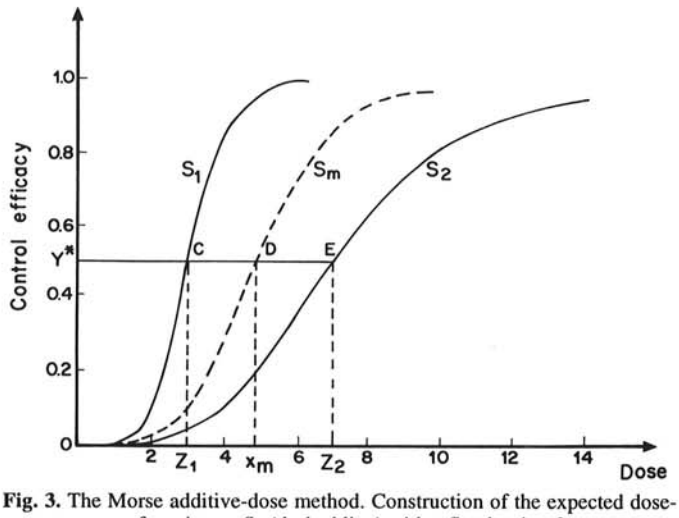

Fig. 3. The Morse additive-dose method. Construction of the expected dose-response curve for mixture S_m (dashed line) with a fixed ratio of components, S_1 and S_2, when the dose-response curves of the constituents (solid lines) are known.

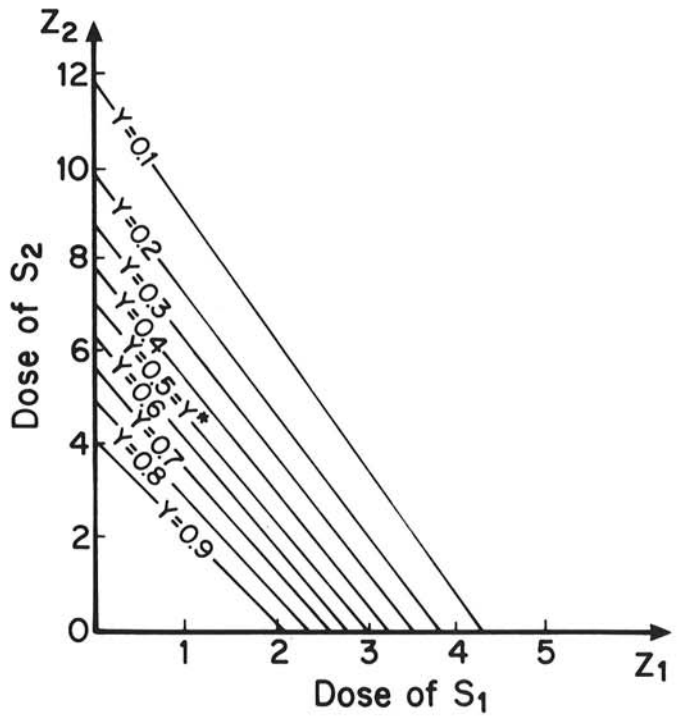

Fig. 4. The "indirect" version of the Morse additive-dose method. The isoboles for different response levels are not parallel.

response curves of its two constituents. Rummens postulated two conditions for an adequate calculation of synergism:

Postulate P1: If two (or more) portions of a single agent are considered together as the mixture, "the technique should identically reproduce the response curve of that agent."

Postulate P2:"When applied to a mixture of two (or more) agents, the calculated response curve should be intermediate between the individual curves for the most effective and the least effective agents at all points."

The second constraint makes sense in the case of mixtures with similar action of constituents, but it is not relevant if action of components is different (Fig. 5).

The expected dose-response curve is determined in the following way.

Step 1: Adjust equation 6 to the experimental data for individual agents and obtain a set of best parameters, b_i,c_i, for each component i ($i = 1,2,\ldots,n$) of the mixture.

Step 2: Calculate the weighted mean values

$$b_e = \pi_1 b_1 + \pi_2 b_2 + \ldots + \pi_n b_n \text{ and } c_e = \pi_1 c_1 + \pi_2 c_2 + \ldots + \pi_n c_n$$

in which $\pi_1,\pi_2,\ldots,\pi_n$ are the fractions of mixture constituents, $\pi_1 + \pi_2 + \ldots + \pi_n = 1$.

Step 3: Substitute parameters b_e and c_e into equation 6 to obtain the equation of the expected dose-response curve for the mixture, and plot this curve. The expected response to any dose of the mixture is read from this dose-response curve.

The Rummens scheme is the direct method. Synergism is defined as the positive deviation of the observed response from that obtained from the expected dose-response curve.

Rummens (29) mentioned the possibility of another, probably more accurate, heuristic procedure, in which the expected dose-response curve for the mixture is obtained by taking the weighted geometric mean for parameter b_e:

$$b_e = b_1^{\pi_1} b_2^{\pi_2} \cdots b_n^{\pi_n}$$

The Abbott Method and the Gowing Formula

The Abbott procedure is the basic approach of the MSM, and it is relevant if different modes of action of mixture constituents are assumed. Let C_1 and C_2 be the effects (proportion of pests killed or disease controlled) of the two components when each is applied alone. This means that the proportions of pests surviving exposure to the first and second pesticides are $q_1 = 1 - C_1$ and $q_2 = 1 - C_2$, respectively. Suppose these two chemicals affect different systems in the pest and act independently, then exposure to the mixture is estimated by the expected value of the proportion of survivors: $q_{exp} = q_1 q_2 = (1 - C_1)(1 - C_2) = 1 - C_1 - C_2 + C_1 C_2$. The expected efficacy, C_{exp}, of the mixture is calculated by the formula

$$C_{exp} = 1 - q_{exp} = C_1 + C_2 - C_1 C_2 = C_1 + C_2(1 - C_1) = C_2 + C_1(1 - C_2) \quad (7)$$

which is sometimes referred to as the Gowing formula (17). Formally, this means that the second component of the mixture controls proportion C_2 of only that part of the pest population, $1 - C_1$, that survived the action of the first pesticide and vice versa.

The Abbott approach is simple in its experimental realization. The efficacies of two pesticides and the mixture consisting of the same doses of those pesticides have to be determined. If the observed experimental efficacy of the mixture is higher than the expected efficacy, a synergistic interaction is declared. The critical point of this procedure is when at least one of the single components is used in the mixture in a relatively high effective dose; this results in a very small deviation of the expected efficacy, which is close to 1, from the observed mixture efficacy, which is also about 1. Therefore, in this case, the Abbott method provides no reliable distinction between synergism and additive action (6, 14,15,22).

The Morse MSM

The MSM of Morse (23) is a graphic representation of the Abbott method. We use the same designations as for the Morse ADM. Like the ADM, Morse constructed the expected dose-response curve for the mixture if the dose-response curves for the components are known. If survival of pests is q_1 and q_2 for doses x_1 and x_2 of components S_1 and S_2, respectively, acting separately, then the model implies that the expected survival for $x_m = x_1 + x_2$ units of mixture S_m is

$$q_m = q_1 q_2 \quad (8)$$

This equation was used for construction of the expected dose-response curve for S_m (Fig. 5) with constant proportions π_1 and π_2 of components S_1 and S_2, respectively, $\pi_1 + \pi_2 = 1$.

To compare expected dose-response curves generated by the MSM and ADM of Morse, an example is shown in Figure 5, in which the dose-response curves for components S_1 and S_2 and proportions π_1 and π_2 in mixture S_m are the same as in Figure 3.

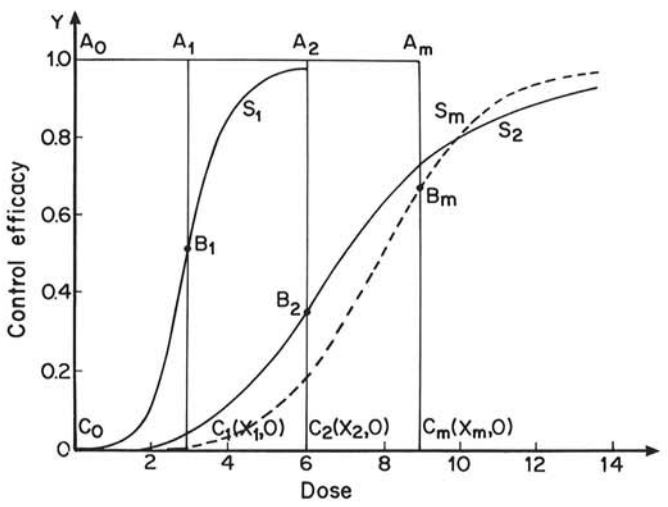

Fig. 5. The Morse multiplicative-survival method. Construction of the expected dose-response curve for mixture S_m (dashed line) with a fixed ratio of components, S_1 and S_2, when the dose-response curves of the constituents (solid lines) are known.

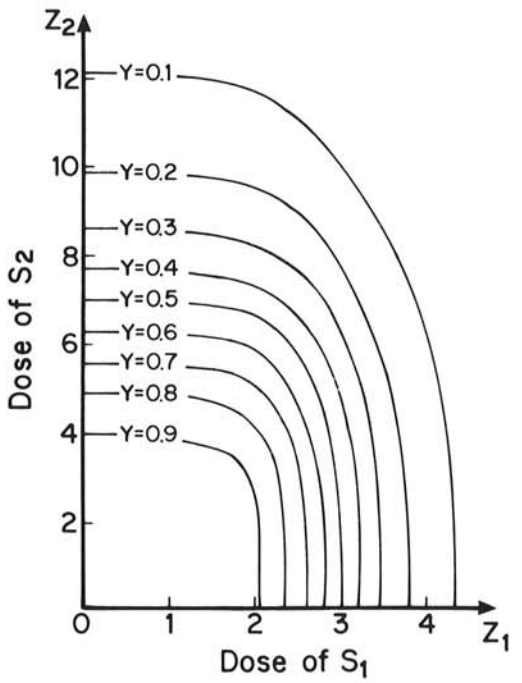

Fig. 6. The "indirect" version of the Morse multiplicative-survival method. The isoboles are not linear.

Locating the dose-response curve for the mixture is realized as follows:

Step 1: Plot the dose-response curves for components S_1 and S_2.

Step 2: Take an arbitrary value, x_m, and calculate $x_1 = \pi_1 x_m$ and $x_2 = \pi_2 x_m$.

Step 3: Draw vertical lines through the points $(x_1,0)$ and $(x_2,0)$ and determine points B_1 and B_2 of the intersections with the curves for S_1 and S_2, respectively.

Step 4: Measure $q_1 = A_1B_1$ and $q_2 = A_2B_2$, and calculate q_m according to equation 8.

Step 5: Draw a vertical line through the point $(x_m,0)$ and locate point B_m (response to x_m units of S_m) by making segment $A_mB_m = q_m$.

Step 6: Repeat the previous steps (2 through 5) for different values of x_m and connect the constructed points to obtain the expected dose-response curve for mixture S_m.

Although the dose-response curves for S_1 and S_2 are the same as in Figure 3 and the ratio $x_1/x_2 = \pi_1/\pi_2$ between the components is unaltered, the expected curves for S_m are substantially different in Figures 3 and 5. The expected dose-response curve for S_m in Figure 5 does not lie entirely between those for S_1 and S_2. Therefore, this curve does not satisfy postulate P2 of the Rummens two-parameter method ([29], and described above). Postulate P1 of Rummens is not fulfilled either (we will discuss this point further).

The direct version of the Morse MSM is realized by comparison of the actual response of x_m units of mixture S_m with the expected response, which is read from the expected dose-response curve. The indirect version of this method is simply realized by the obtained expected curve for mixture S_m (Fig. 5) with a constant ratio, π_1/π_2, of the components.

The isoboles of the Morse MSM are shown in Figure 6. They can be constructed for an arbitrary fixed level of control, $Y^\star$, by the following procedure:

Step 1: Take x_1 units of component S_1, which provides control Y_1 ($Y_1 \leq Y^\star$).

Step 2: Determine x_2 units of component S_2 (from the dose-response curve for S_2 in Fig. 5), which provides control $Y_2 = 1 - (1 - Y^\star)/(1 - Y_1) = (Y^\star - Y_1)/(1 - Y_1)$, $Y_2 \leq Y^\star$.

Step 3: Plot the point with coordinates (x_1,x_2) on the plane against two perpendicular axes "dose of S_1" and "dose of S_2."

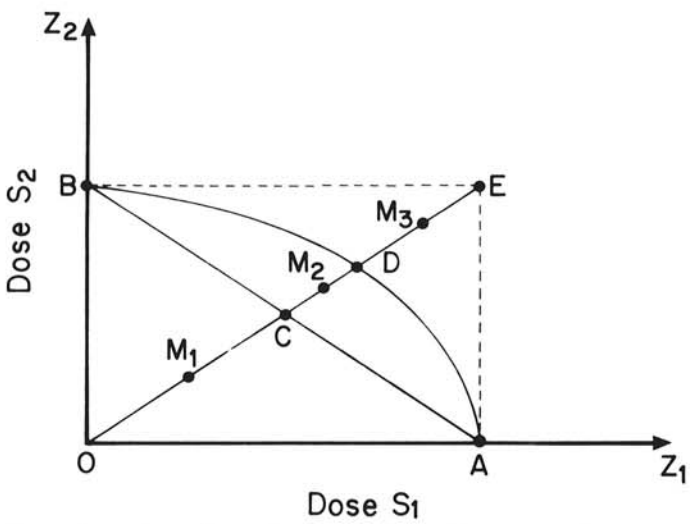

Fig. 7. Comparison of the Abbott and Wadley methods. Straight line *AB* and concave downward curve *ADB* are the isoboles of Wadley and Abbott, respectively, for a given response level. Points M_1, M_2, and M_3 correspond to three doses of S_m that actually provide this level of response. For M_1 (M_3) both methods indicate synergism (antagonism), although the Abbott (Wadley) approach indicates a stronger synergistic (antagonistic) interaction than that of Wadley (Abbott). For M_2 a synergistic interaction is declared according to Abbott, and antagonism is declared according to Wadley.

Step 4: Repeat steps 1 through 4 for different values of x_1 and connect the constructed points to obtain the isobole corresponding to the initial level of response $Y^\star$. The Morse MSM gives graphic representation (isoboles) of the Abbott approach. The isoboles of any MSM are not linear.

Comparison of the ADM and MSM

The Abbott approach indicates stronger synergistic interaction compared to the Wadley approach if they are applied to the same data (15). This becomes clear from comparison of the isoboles of Wadley (Fig. 1) and Abbott (Fig. 6). Let us consider Figure 7, in which the Wadley isobole (straight line *AB*) and that of Abbott (concave downward curve *ADB*) for any given response level, *Y*, are drawn. The ray through origin *O* corresponds to various doses, $x_m = x_1 + x_2$, of a mixture, S_m, comprising components S_1 and S_2 in a constant ratio, $x_1/x_2 = const.$ Points *A* and *B* correspond to the doses of S_1 and S_2, respectively, which when applied alone result in response *Y*.

Points *C* and *D* correspond to the expected doses of mixture S_m, which provide the response of *Y*, if they are determined by the Wadley and Abbott approaches, respectively. Three possibilities exist for the observed dose of S_m, which actually provides this level of response. They are expressed by the arbitrary points M_1, M_2, and M_3 from intervals *OC*, *CD*, and *CE*, respectively, with coordinates that correspond to the doses of the components making up the corresponding mixtures. The intensity of synergistic (or antagonistic) interaction, defined by the cotoxicity ratio between the expected and the observed effective doses for a fixed level of control, is geometrically expressed by the formulas

$$Int_W = OC/OM_i \text{ and } Int_A = OD/OM_i$$

for the Wadley and Abbott schemes, respectively, in which $i = 1,2,3$. Therefore, if point M_1 corresponds to the actual dose, both methods declare synergism, although the Abbott approach indicates a stronger synergistic interaction than does the Wadley approach, because $Int_A > Int_W > 1$ ($OD > OC$). On the other hand, $OD < OE = 2OC$ and $Int_W < Int_A < 2Int_W$. Therefore, the cotoxicity ratio of Abbott theoretically can exceed that of Wadley by no more than two times. To compare intensities of interaction according to Abbott and Wadley, corresponding cotoxicity ratios or synergy ratios must be used. It is incorrect to compare the cotoxicity ratio of any mixture with the synergy ratio of another one, because these two measures are inconsonant.

If point M_3 (Fig. 7) corresponds to the actual dose, both methods indicate antagonism, although the Wadley approach indicates a stronger antagonistic interaction than does the Abbott approach, because $1 > Int_A > Int_W$.

The discrepant conclusions are pronounced if point M_2 between *C* and *D* (Fig. 7) corresponds to the actual dose. The interaction is synergistic according to Abbott, because $Int_A > 1$, and antagonistic according to Wadley, because $Int_W < 1$. This wide difference does not mean that one method is more correct than the other. Each is derived from distinct assumptions of either similar or different action of the components. Choosing a suitable method for any given situation is a key step in investigating synergism.

To formally explain why the Abbott method is inappropriate in the case of similar action and to make clearer the difference between ADM and MSM, we consider a mixture that consists of two equal portions, *d*, of a single component, *S*. Control efficacy *C* of $z = d + d$ units of the mixture is estimated. The mixture components are of similar action, and it is natural to request that the expected efficacy of this mixture, $C(z)$, equals the efficacy, $C(2d)$, of $2d$ units of *S*. If the Abbott approach is exploited, the expected efficacy of the mixture is $C(z) = C(d) + C(d) - C(d)C(d)$ and, hence, $C(2d) = C(d) + C(d) - C(d)C(d)$. Based on the same reasons, if two portions of nd (for any integer, n) and d units of component *S* make up the mixture, then the following equation holds:

$$C[(n+1)d] = C(nd) + C(d) - C(nd)C(d) \text{ and } C(d) = C_0 \qquad (9)$$

in which C_0 is the efficacy of the dose, d. Equation 9 is the recursion formula, which determines virtually the equation of dose-response curve for component S: $C(x) = 1 - (1 - C_0)^x$.

This curve depends only on parameter C_0, which results in the following unexpected conclusion: if two dose-response curves for two different agents cross each other, then these curves coincide identically. This is denied by actual data of control efficacy of different pesticides, and therefore, it is wrong. This implies that the Abbott method is inappropriate in the case of similar action of mixture constituents.

If trials of the mixture and its constituents are adequately replicated, determination of the significance of a synergistic interaction is possible. Finney (9) outlined a χ^2 test to compare the actual effect of a mixture with the expected effect. However, collecting a large amount of replicated data under identical conditions may be difficult.

Biometrical Models of Joint Action

The empirical methods of measuring joint action of pesticides are simple and convenient to use. However, they do not provide suitable statistical control to evaluate the significance of the results. A few biometrical models of joint action were developed to solve this problem. Unfortunately, experimental testing of these models is relatively scanty, and results often contradict the biological facts (3). Therefore, the biometrical models have not been established in research practice. We only list them; the reader is referred to Banki (3) for more information.

The Bliss (4) and Finney models (12) were developed under the principal assumption that both components and the mixture exhibit parallel dose-response straight lines after the log-probit transformation. Three types of joint action were separately considered for the estimation of the expected response to any mixture: (i) similar action, when the effect of the bioactive agents is identical with respect to the site of action within the pest; (ii) independent action, when modes of action are different; and (iii) synergistic (or antagonistic) action, for situations different from i and ii. It was assumed that two constituents do not influence each other's action in i and ii.

The Robertson-Smith model (28) was developed for testing the hypothesis of independent uncorrelated action of mixture components with "parallel dose-response lines." The dose of the mixture required for 50% control was predicted from the model and divided by that from the probit line fitted to the mixture. If this cotoxicity ratio is greater or less than 1, then synergism or antagonism, respectively, was declared. The assumptions of parallelism and the large number of identical replications of experiments needed make these three models inappropriate for practical use.

The theory of Plackett and Hewlett (21,25) is based on the classification of biological relations of joint action according to the mode of action of the pesticides and on parameters characterizing the mechanism of such action (Table 17 in reference 3). Mixture components were divided into two groups of similar and dissimilar modes of action depending on the final target affected by each constituent. Interactive and noninteractive agents were considered with regard to whether one of the components modifies the physiological action of the other component. The model parameters are the correlation of the susceptibility of the pest to the components, the slope of the dose-response curves, and a parameter relevant to the transition of the mode of action. The Plackett-Hewlett model is complex. A relatively simple explanation of this model with minimum mathematical formalism was done by Banki (3). Unfortunately, like the other biometrical models, the practical value of the Plackett-Hewlett theory is questionable.

Improved Empirical Methods of Joint Action

We have developed (discussed below) new methods for estimating the expected efficacy of a mixture according to the proposed classification of joint action of pesticides. We assume that the efficacy of a pesticide depends on the density of a pest population that was proved for fungicides by Samoucha et al. (31).

We use the following designations: d_M, d_A, and d_B are doses of mixture M and its constituents A and B, respectively, $d_M = d_A + d_B$; $C(d_A)$ and $C(d_B)$ are the control efficacies of d_A and d_B applied alone, respectively. We assume that a similar level of inoculum (=initial density of pest population) is used for plants treated with either the mixture or the single pesticides and that the dose-response curves for pesticides A and B have an S shape.

Different Simultaneous Action of the Mixture Constituents

The effect of dose d_A of pesticide A or dose d_B of pesticide B results in pest survival with a probability (percentage of disease) of $1 - C(d_A)$ and $1 - C(d_B)$, respectively. The independent action of pesticides is assumed, because different targets are suppressed by each component. The expected probability of pest survival is equal to $[1 - C(d_A)][1 - C(d_B)]$. This implies that for the direct version the following equation describes the expected efficacy:

$$C_{exp}(d_M) = 1 - [1 - C(d_A)][1 - C(d_B)] = C(d_A) + C(d_B) - C(d_A)C(d_B) \quad (10)$$

which is the Abbott estimation (equation 7). The indirect version is, in fact, that of the Morse MSM (Fig. 6).

Similar Simultaneous Action of the Mixture Constituents: The Double-Replacement Method

Let two pesticides affect the same target in a pest, and their dose-response S-shaped curves may be nonparallel after log-probit transformation (Fig. 8A and B). As shown in Figure 9, the relation between equivalent effective doses of mixture constituents may be variable and, therefore, using the Wadley procedure to estimate the expected efficacy of the mixture would be inappropriate. A new approach is proposed to assess the expected efficacy of a mixture irrespective of the form of dependence between equipotential doses of the components.

Let $de_B(d_A)$ and $de_A(d_B)$ denote the equivalent effective dose of pesticide B with respect to dose d_A of pesticide A and the equi-

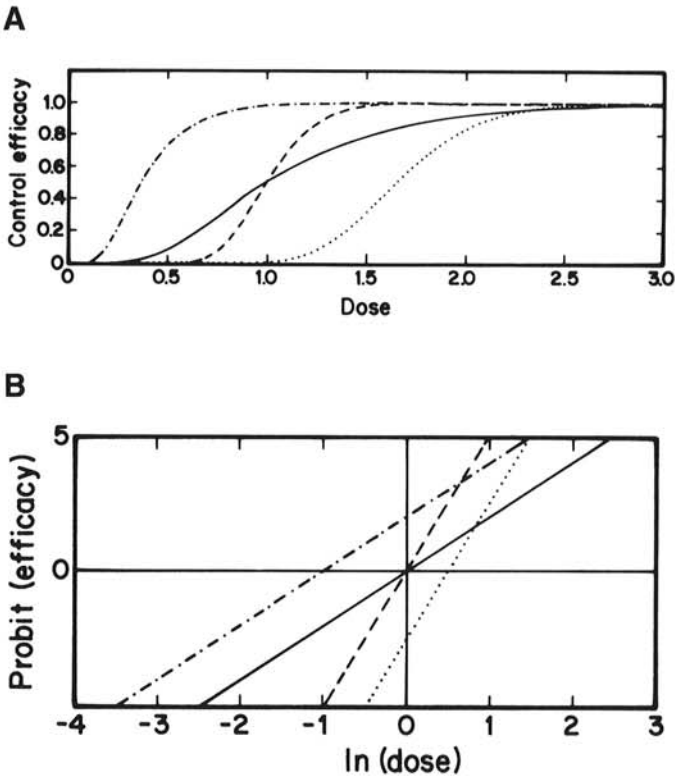

Fig. 8. Dose-response curves of different pesticides **A,** fitted to log-normal form, and **B,** after log-probit transformation. Dose-response S-shaped curves may be nonparallel after log-probit transformation.

valent effective dose of pesticide A with regard to dose d_B of pesticide B, respectively. The direct version of the new method for assessing the expected efficacy of any given mixture, $d_M = d_A + d_B$, should follow this procedure:

Step 1: Plot the experimental dose-response curves of pesticides A and B (Fig. 10).

Step 2: Determine the equivalent dose, $de_A(d_B)$, of A for dose d_B of B (Fig. 10): $d_B = 0.4$, $C(d_B) = C(d_A) = 0.57$, and $de_A(d_B) = 1.1$.

Step 3: Substitute equivalent dose $de_A(d_B)$ of A for dose d_B of B in the mixture. The dose, $de_A(d_M) = d_A + de_A(d_B)$, of A can be considered as equipotential to dose $d_M = d_A + d_B$ of the mixture: $d_A = 0.9$ and $de_A(d_M) = 0.9 + 1.1 = 2.0$.

Step 4: Determine the efficacy of dose $de_A(d_M)$ of A when applied alone (Fig. 10). One possible estimation of the mixture efficacy is $C[de_A(d_M)]$: $C[(de_A(d_M)] = 0.88$.

Step 5: Repeat steps 2 through 4, in which A is replaced by B and vice versa. The dose, $de_B(d_M) = de_B(d_A) + d_B$, of component B can be considered as equipotential to the dose, $d_M = d_A + d_B$, of the mixture. Another possible estimation of the mixture efficacy is the effect of dose $de_B(d_M)$ of B when applied alone, $C[de_B(d_M)]$.

Step 6: Select the maximum (or minimum) value among $C[de_A(d_M)]$ and $C[de_B(d_M)]$ as the expected efficacy of the mixture to reveal synergism (or antagonism).

If one ingredient of the mixture can be replaced by the other at a constant rate, then predicted values $C[de_A(d_M)]$ and $C[de_B(d_M)]$ are equal, and a single estimation of the expected efficacy is obtained. Generally, the values of predicted mixture efficacy are different, depending on the chosen replacement of the mixture constituents. To obtain a reliable conclusion about synergism (or antagonism), the maximum (or minimum) of these two values predicted from the double replacement should be taken as the expected efficacy of the mixture.

The indirect version of this method, which results in construction of regions of synergistic and antagonistic interaction of mixture constituents, should follows these steps:

Step 1: Plot dose-response curves for pesticides A and B (Fig. 11A).

Step 2: Choose the level of efficacy you want (e.g., 0.9).

Step 3: Determine $ED_{0.9}B$ (Fig 11A).

Step 4: Choose an arbitrary dose, d_B, of pesticide B.

Find the complementary dose, d_A, of A that together with d_B provides efficacy 0.9 using additional steps:

Step 5: Find the complementary dose, d'_B, of pesticide B that together with d_B provides efficacy 0.9, $d'_B = ED_{0.9}B - d_B$.

Step 6: Look for the dose, $de_A(d'_B)$, of A that provides the same level of control as d'_B (Fig. 11A). Dose $d_A = de_A(d'_B)$ complements d_B to provide efficacy 0.9.

Step 7: Plot a point with coordinates (d_B,d_A) (Fig. 11B). This point together with other similarly obtained points (for other values of d_B between 0 and $ED_{0.9}B$) produce the first boundary line of the "regions of joint action" (lower line in Fig. 11B).

Step 8: Repeat this process for various doses, d_A, of A (between 0 and $ED_{0.9}A$), and plot the second boundary line of the regions of joint action (upper line in Fig. 11B).

Determination of the type of interaction of the mixture components is implemented as for Wadley's isoboles. If the "experimental" point falls below (or above) both boundary lines of the regions of joint action (Fig. 12), the mixture is synergistic (or antagonistic) for the given fixed control level. If the experimental point falls between the boundary lines, no reliable conclusion can be drawn.

The intensity of the synergistic (or antagonistic) interaction is determined as with the Wadley method. The lower (or upper) boundary line (Fig. 12) is used instead of the straight line of Wadley's expected isobole (Fig. 1). The mixed proportions required to achieve the desired level of pest or disease control with minimum total amount of components is optimal when a mixture exhibits the maximal intensity of synergistic interaction. Thus, when the observed effective doses of mixtures made up of different proportions of the components are plotted, the optimal proportion can be obtained.

This method enables accurate analysis of interaction between pesticides whose dose-response curves are of any S shape. No additional constraints are assumed. When the dose-response curves of the components are parallel after log-probit transformation, the two boundary lines coinside with the same straight line, and the region of uncertainty vanishes (Fig. 13). The procedure proposed is reduced to the Wadley method when the dose-response curves are "parallel."

Nonsimultaneous Action of the Mixture Constituents

Mixtures of fungicides often consist of one component that affects fungal spore germination and another that affects mycelial

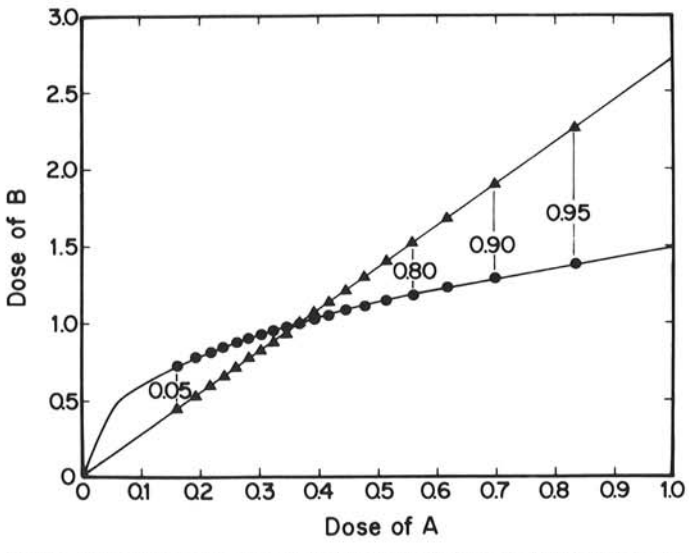

Fig. 9. Dependence between doses of equal efficacy for two pairs of pesticides. The straight line corresponds to the solid (**B**) and dash-dotted (**A**) dose-response curves in Figure 8, which are parallel after log-probit transformation. The curved line corresponds to the dashed (**B**) and dash-dotted (**A**) dose-response curves in Figure 8, respectively, which are not parallel after log-probit transformation. Triangles and circles correspond to efficacy levels 0.05, 0.10, ..., 0.90, and 0.95 for given pesticides.

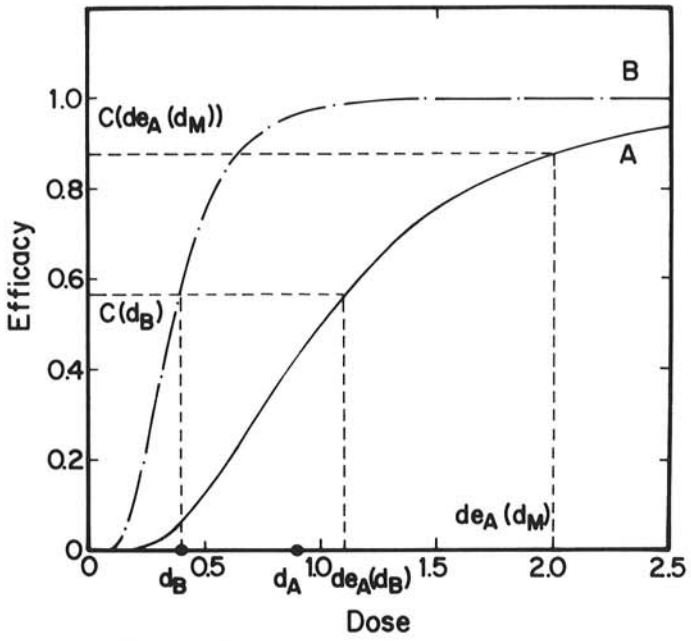

Fig. 10. The "direct" version of the new method. The expected efficacy of the mixture, $d_M = d_A + d_B$, in which d_A and d_B are doses of components A and B, respectively, is determined. One possible estimation of the mixture efficacy, $C[de_A(d_M)]$, is obtained when B is replaced by A.

growth without affecting spore germination. Let us assume that pesticide B begins its action against a pest with some delay relative to pesticide A. The effect of dose d_A of A results in control of the $C(d_A)$ portion of the initial pest population. Pesticide B, beginning later, suppresses the residual part of the population, $1 - C(d_A)$, that was not affected by A. No overlapping is assumed to occur in the action of the mixture components. (Overlapping is evident when the pesticides act simultaneously, so both can suppress the same individuals even though one is enough to do so.)

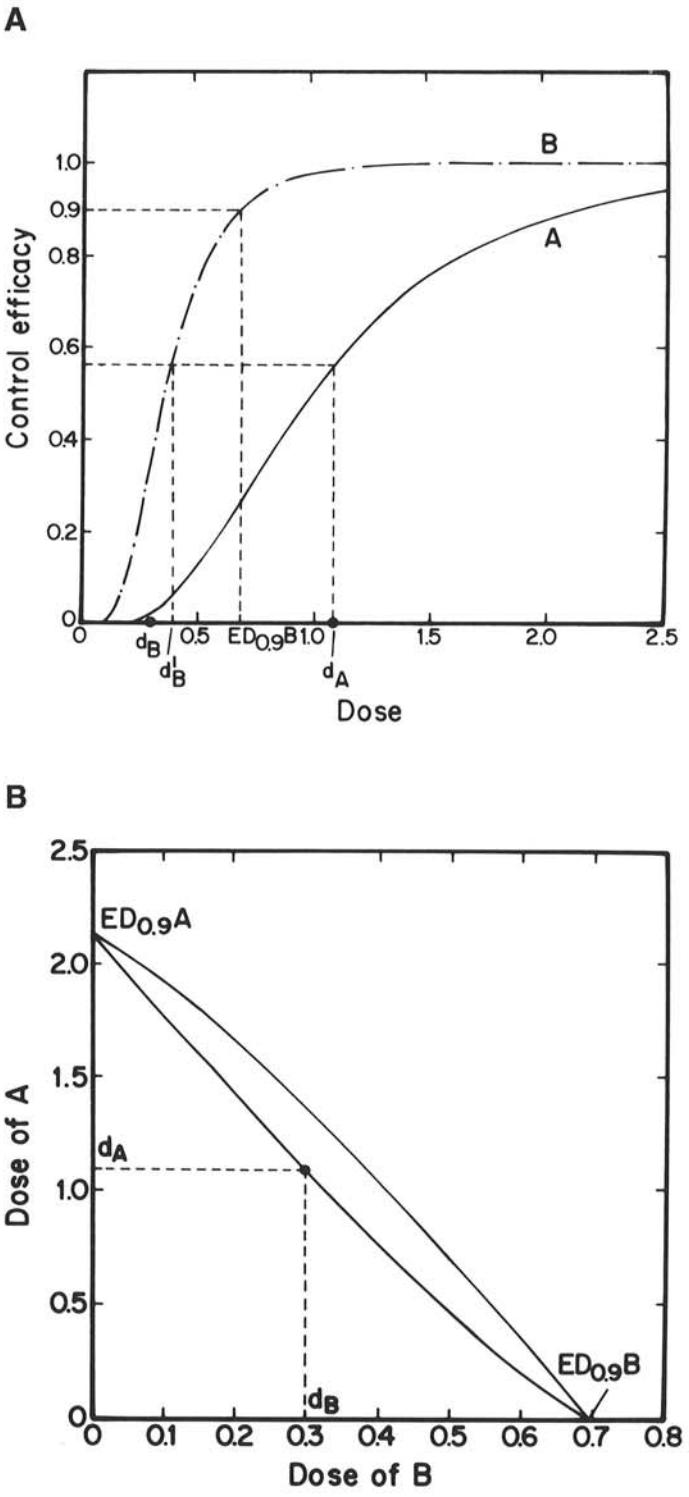

Fig. 11. Boundary lines of regions of joint action. **A,** Determination of mixture $d_M = d_A + d_B$ at efficacy level 0.9 (point on the first boundary line) when component A is replaced by B. **B,** Construction of the first (lower) boundary line. The second boundary line is constructed in a similar way.

The following algorithm (the direct version) is proposed to estimate the expected efficacy of the mixture comprising pesticides with nonsimultaneous action.

Step 1: Measure the efficacy, $C(d_A)$, of dose d_A of pesticide A used alone for controlling a fixed level of initial pest population.

Step 2: Determine in another experiment the efficacy, $C'(d_B)$, of dose d_B of pesticide B in controlling a pest population whose density equals $1 - C(d_A)$ of that fixed level.

Step 3: Calculate the expected efficacy of the mixture as follows:

$$C_{exp}(d_M) = C(d_A) + C'(d_B)[1 - C(d_A)] = C(d_A) + C'(d_B) - C(d_A)C'(d_B) \quad (11)$$

This formula reflects the fact that the effects of pesticide A and, after that, the effects of pesticide B are incompatible events. This implies that the expected probability to control a pest population by the mixture is equal to the sum of the probabilities of suppressing the pest with doses d_A and d_B of the constituents. These probabilities are actually the efficacies of the corresponding doses (the conditional efficacy for pesticide B) and are equal to $C(d_A)$ and $C'(d_B)[1 - C(d_A)]$ for pesticides A and B, respectively, if B acts after A.

Equations 10 and 11 differ only in the efficacy of dose d_B. Samoucha et al. (31) showed that the efficacy of a fixed dose of a

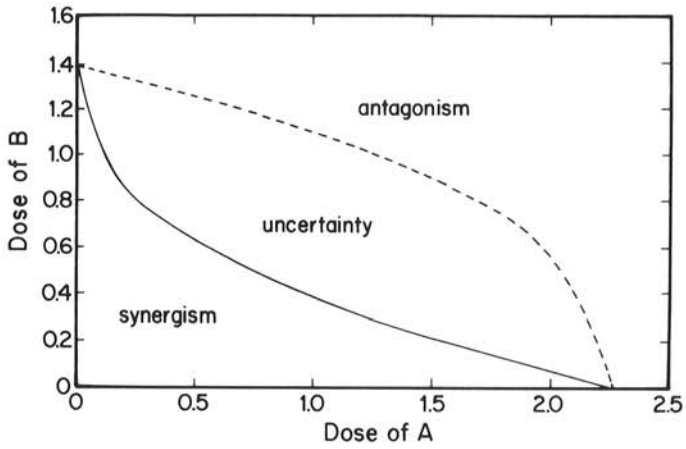

Fig. 12. Regions of synergistic and antagonistic interaction.

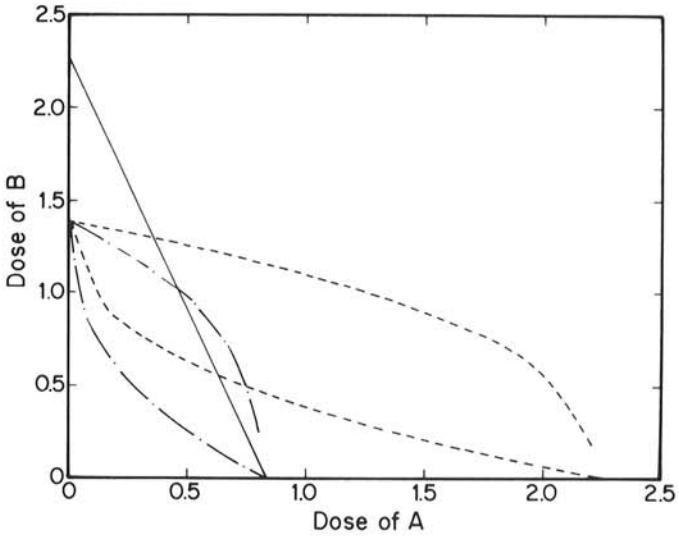

Fig. 13. Regions of joint action for different mixtures. The efficacy level is 0.95. The solid boundary line corresponds to the solid (**B**) and dash-dotted (**A**) dose-response curves in Figure 8. The dashed boundary lines correspond to the solid (**A**) and dashed (**B**) dose-response curves in Figure 8. The dash-dotted boundary lines correspond to the dash-dotted (**A**) and dashed (**B**) dose-response curves in Figure 8.

fungicide increased when the initial inoculum density was diminished. Therefore, it is natural to suppose that $C'(d_B)$ is not smaller than $C(d_B)$. This has important consequences.

Let us consider two mixtures, M_1 and M_2, that consist of pesticides A_1 and B_1 and A_2 and B_2, respectively. Let these pesticides meet the following conditions: (i) A_1 and B_1 are characterized by different simultaneous action; (ii) A_2 and B_2 are characterized by nonsimultaneous action; (iii) A_1 and A_2, B_1 and B_2 are taken in equal effective doses of d_{A_1} and d_{A_2}, d_{B_1} and d_{B_2}, respectively, in mixtures M_1 and M_2 i.e., $C(d_{A_1}) = C(d_{A_2})$ and $C(d_{B_1}) = C(d_{B_2})$. The expected efficacy of dose $d_{M_1} = d_{A_1} + d_{B_1}$ of mixture M_1 is not greater than that of dose $d_{M_2} = d_{A_2} + d_{B_2}$ of mixture M_2, i.e., $C_{exp}(d_{M_1}) \leq C_{exp}(d_{M_2})$. This means that adjusting Abbott equation 10 to a mixture made up of components with nonsimultaneous action may result in underestimation of the expected efficacy and, hence, in overestimating the synergy ratio (equation 1). More thorough analysis of nonsimultaneous action reveals that partial overlapping may occur in the action of the mixture components on individuals in the pest population. It is unrealistic to estimate this overlapping and calculate the actual expected efficacy of such a mixture. However, this expected efficacy is always less than the estimate of equation 11 and greater than that of Abbott (equation 10). Hence, the upper limit of the expected efficacy (equation 11) must be used for reliable conclusions.

The indirect version of this method is possible, but difficult, to implement.

CONCLUDING REMARKS

(i) The empirical methods for analyzing the joint action of pesticides in mixture are simple and convenient to use. The basic problem is whether the mode of action of mixture constituents is similar. This determines what procedure should be chosen, either ADM or MSM, for estimating the expected effect of any specific mixture. If the biological mode of action is unknown, several mathematical methods (including at least one of the MSM and ADM) must be applied correctly to specific data. The interaction between mixture components is synergistic (or antagonistic) when it is confirmed by all tests applied.

(ii) The synergism demonstrated for a certain ratio of components cannot be extrapolated for another ratio unless it is determined in an independent experiment.

Both conclusions are crucial for reliable proof of a synergistic effect produced by combinations of pesticides (27).

(iii) All methods discussed in this paper may be extended for mixtures of three or more constituents. This has been done already with the Abbott (7), Wadley (18), and Rummens (29) methods. With other methods, such extension is complicated.

(iv) Joint action of pesticides as a function of time after spray or inoculation and under field conditions was not discussed in this paper. This problem is of significant practical and theoretical importance, but its complexity makes a comprehensive solution difficult. Only partial attempts have been made to elaborate on these problems (3), and they are still far from being solved. Corresponding models of pest development probably will have to be used to achieve any progress in investigation of time- and environment-dependent interactions of pesticides.

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