0031-6997/89/4102-0093$02.00/0
PHARMACOLOGICAL REVIEWS
Vol. 1989, No. 41
Copyright © 2 by The American Society for Pharmacology and Experimental Therapeutics
Printed in U.S.A.

What is Synergy?

M. C. Berenbaum

Department of Experimental Pathology, St. Mary's Hospital Medical School, London, W2 1PG, United Kingdom

I. Introduction 93
II. Expectation: Mechanistic and empirical models 95
III. Terminology and definitions 99
IV. Parameters for assessing interactions 99
V. The Isobole Method 100
A. General validation of the method 100
B. Remarks on the isobole method 101
1. Data required for assessing interactions 101
2. Isoboles are not necessarily consistent or similar 103
3. Statistical examination of isoboles 103
VI. Calculations of the expected effect of a combination 104
A. The general case 104
B. Particular cases 105
1. Linear dose-response curves. Effect summation 106
2. Simple exponential dose-response curves. Survivor multiplication 106
3. Exponential dose-response curves with shoulders. Similar curves 108
4. Exponential dose-response curves with shoulders. Dissimilar curves 108
5. Linear-log and log-log dose-response curves 108
6. Sigmoid dose-response curves. 108
i. Mutually exclusive agents. The median effect principle 108
ii. Mutually nonexclusive agents 111
iii. Curve-fitting 113
iv. Application of the mass-action law in complex biological systems 114
v. Utility of the median effect principle 115
VII. Additivity envelopes 116
VIII. Similar and dissimilar action. Independence and zero interaction. Concentration- and response-addition. 117
IX. Some important aspects of interactions 122
A. Significance of minor interactions 122
B. Agent multiplicity 123
X. Therapeutic synergy. Multifunctional interactions 123
XI. Finding the optimum 126
A. Response surface modelling 126
B. Direct search methods 127
XII. Finding the minimum. Complex environmental mixtures 129

I. Introduction

INTERACTIONS between biologically active agents are important for several reasons.

1. Combinations of agents are used clinically for the therapeutic advantages they may provide over single agents. The best evidence for these advantages comes from the treatment of malignant disease and of infections. There is little doubt that the use of combinations of cytotoxic drugs has increased survival in patients with leukaemias, lymphomas, and some other neoplasms, so that the current preference in these conditions is for regimens with 4–6 or more agents (61, 70, 81, 92, 143, 184, 209, 210, 214, 460, 546).

For instance, in acute lymphatic leukaemia of childhood, the percentage of remissions with single agents is 40–50%, as compared with 94–95% with combinations of three drugs (460). Again, in small cell carcinoma of the lung, treatment with single agents produces complete remission in only 2.5% of patients, compared with 19–25% in those receiving combinations of two to four drugs

UNITED THERAPEUTICS CORP., EX1034, page 1
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

(81,214). Several examples from the earlier literature were reviewed by Henderson and Samaha (242).

Combinations of antibiotics are useful in the treatment of serious infections (141, 163, 181, 297–301, 363, 495, 552, 558), especially when these combinations have been shown to be synergistic in vitro (7, 297–301, 368, 360, 433, 501). For example, Anderson (7) found that the response rate in neutropenic patients with gram-negative infections was 33% if the antibiotic combination was not synergistic in vitro and 79% if it was. In patients with *Pseudomonas* infections, the corresponding figures were 0% and 84%. Klastersky and Zinner (301), reviewing several papers, found that, in patients with a variety of infections, the cure rate was 0–49% (mean 43%) if the combination was not synergistic and 48–88% (mean 76%) if it was.

Drug combinations may also be advantageous in, for example, the treatment of cardiac failure (415a, 467, 496), myocardial infarction (122–124, 266), severe hypertension (369) (in which about 60–70% of patients require combinations of two or three drugs (416)) and asthma (48, 73, 134, 259, 327, 452), and in the prevention of graft rejection (461).

2. Most patients who receive drug combinations do so, not for any supposed advantage afforded by the combination, but because several therapeutic indications are present. Hospitalised patients may receive a surprising number of different drugs. May (359) found, in the early 1970's, that patients received an average of 7.9 drugs during their stay in a general hospital, and Cluff (119) found that patients given an antibiotic received on average 13 additional drugs. The number and variety of drugs received by comparable patients today is unlikely to be less. Such multiple drug-taking is not confined to hospital patients; recent surveys showed that, at any one time, 20% of elderly persons in the community are taking three or more prescribed drugs, and this proportion rises to over 50% for patients referred to a geriatric service (94, 206). Although such combinations are not given in order to obtain an interaction, one may nevertheless occur, and such inadvertent interactions are more often deleterious than beneficial. Stockley (484) lists about 600 known interactions between commonly used drugs, almost all adverse. For example, aminoglycosides in combination with cephalothin may be nephrotoxic, aminoglycosides together with ethacrynic acid may be ototoxic, the anticoagulant effects of warfarin are increased by cimetidine, the antihypertensive effects of guanethidine may be abrogated by tricyclic antidepressants, and so on. May (359) found that over 50% of hospital patients given 16–20 drugs had adverse effects serious enough to require treatment or a change of therapy and, while most such effects are probably due simply to excessive or inappropriate prescribing, about 20–25% may be due to drug interactions (62). Among elderly, non-hospitalized individuals, 17–20% are taking combinations of drugs with the potential for harmful interactions (94,206).

3. Governmental regulations as to permitted levels of toxic environmental pollutants generally refer to single agents and do not explicitly take into account the possibility that they might interact in producing harmful effects. Nevertheless, there is good evidence that such interactions occur and are important. For example, individuals exposed to more than one human carcinogen have a greater than expected incidence of cancer (58, 418, 435, 446–448), and it has been proposed that the difficulty of accounting for the incidence of some neoplasms in individuals exposed to low-level ionising radiations may be due to hitherto unsuspected synergy with other environmental agents (75). Soil and plants in industrial areas may have raised levels of eight different metals and, in view of the known carcinogenicity of many of these in man or laboratory animals (25a, 271), a connection between this and the raised incidence of lung cancer in such areas has been suggested (330, 331, 464). It has also been argued that the high incidence of neurological and psychometric impairment in individuals occupationally exposed to mixtures of 8 to 10 neurotoxic organic solvents, as compared with the low incidence in those exposed to higher total levels of single solvents, may be due to synergistic interactions (165, 229, 265, 328, 488), a possibility that is supported by animal experiment (389). Simmons et al. (458) found that the hepatotoxicity in mice of complex industrial wastes (which contained 10 to 12 known toxic compounds) could not be accounted for by the toxicities of their known components and suggested that synergistic interactions might, at least in part, be responsible for this discrepancy. There are serious difficulties in attempting to analyse interactions between the constituents of the complex mixtures present in the environment (101, 125, 199, 323a, 358, 483, 554, 555) and these are discussed below (section XII).

Interactions between other adverse industrial factors are the subject of increasing interest (59, 244, 350, 390), and there is a substantial literature on the analysis of interactions in epidemiology (57, 192, 347, 423, 424, 436, 455, 500, 525, 534). These examples show that the public health implications of interactions between environmental agents, and especially the effects of agent multiplicity (43), require urgent and serious consideration.

4. Finally, it seems highly likely that many physiological and pathological processes are governed by interactions (synergistic and antagonistic) between biological mediators (growth factors, interferons, hormones, mediators of inflammation, clotting factors, etc). Such processes include cell proliferation (11, 74, 76, 77, 82, 87, 139, 146, 148, 158, 164, 169, 208, 257, 284, 305, 343, 344, 361, 372, 399, 414, 426–428, 441), cell differentiation and synthetic activities (63, 72, 84, 95, 102, 147a, 159, 191, 218, 282, 343, 393, 429, 440, 462, 535, 536, 564), embry-

UNITED THERAPEUTICS CORP., EX1034, page 2
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

onic induction (288a, 460a), platelet activation and thrombosis (15, 136, 197, 213, 236, 280, 381, 455a, 481, 482, 489, 528), thrombolysis (122–124, 319, 477a, 562), acute inflammation (12, 13, 27, 28, 32, 33, 139, 241, 402, 475, 530, 542–544), shock (391, 520), immunological responses (49, 55, 78, 82, 166, 168, 173, 174, 196, 221, 364, 394, 419, 478, 549), hormone release and hormone action (5, 10, 34, 207, 325, 380, 382, 386, 396, 421, 438, 470), renal water and sodium excretion (9, 199a, 417), muscle catabolism (179), bronchoconstriction (31, 32, 48, 188, 320, 321, 403), and vasoconstriction and regulation of blood pressure (228, 275, 281a, 379). These widespread interactions may have teleological significance. In synergistic interactions, the cooperation of several agents may be needed for a full effect, ensuring that important physiological processes are not made fully operational without important reasons, whereas antagonistic interactions may serve to limit the extent to which these processes operate.

Thus, interactions between biologically active agents are unquestionably of great importance in therapeutics, toxicology, environmental studies, and normal and abnormal physiology. It is therefore a matter for concern that the analysis of such interactions is in a confused state. The concern is the greater in that conclusions based on confused and faulty premises may be used to guide action, for instance, in planning clinical trials, setting environmental standards and so on.

Interactions are generally described as being synergistic or antagonistic. Synergy means, broadly, "working together" and antagonism means "working against each other," and these terms imply the existence of some intermediate, zero-interactive state in which agents do neither of the above. However, these generally understood meanings are not unambiguous definitions; some quantitative criterion is clearly implied, but there does not seem to be any general agreement about what this is.

This unresolved issue does not seriously affect those studies on interactions which are concerned with combinations of agents where the effect of interest is produced by one of the agents only, and in which the other(s) act merely to increase or decrease the effect of the former, for instance, by modifying its absorption, metabolism, or excretion. These so-called heterergic interactions are discussed in section V B 1 (b) below. In the absence of interactions, the effect of such a combination would simply be that of the active agent. When it is greater or less than this, there is no difficulty in deciding that one agent is working together with or against the other, i.e., that synergy or antagonism is present. Problems arise, however, when each agent on its own (or, in multi-agent combinations, more than one of the agents) can produce the effect under study. One would intuitively expect the effect of such a combination to exceed the effect of any one of its constituents, but there is no consensus on how to distinguish between this expected increase and a true interaction.

II. Expectation: Mechanistic and Empirical Models

Before examining the various solutions to this problem that have been proposed, we consider first the idea that the nature of an interaction might be decided by analysing the mechanisms of action of the agents (and/or constructing hypotheses about these mechanisms) and, on this basis, calculating the expected effect of the combination. For example, one might believe that the effects of agents A and B are each adequately explained by binding to some biologically important substrate. With sufficiently good quantitative information (and/or hypotheses) about factors such as the reversibility or otherwise of binding, the number of binding sites, whether A and B bind to the same or different sites, and so on, one might construct an equation describing the expected effects of the agents and their combinations. Then, according to this appoach, zero interaction is defined simply as that state in which the observed effects fit the equation, and synergy and antagonism are defined as divergence between observation and expectation.

The fundamental difficulty here in the context of analysing drug interactions is that the decision as to whether the observed effect of a combination is that which is expected from the mechanisms of action of the agents or unexpected depends entirely on the current state of knowledge, which is largely an accidental and temporal consideration. If mechanisms of action are not well understood or if the model is inappropriate, the effect of a combination of agents may well be unexpected. Then, with increasing understanding of the agents, the model is progressively modified so as to fit the observed effect, and the effect becomes what is expected. For instance, using the above example, it might be believed that A and B have common binding sites, and an equation for the expected effects of their combinations is derived on this basis. If it is observed that the combinations are more effective than the equation predicts, then A and B are claimed to show synergy. Suppose that it is subsequently found that the binding sites are in fact different, and this new information leads to the derivation of a different equation. If the effects are found to fit this new equation, A and B will now be said to show zero interaction. Thus, "interactions" defined in this way vanish as knowledge expands. Moreover, whatever opinion is formed about an interaction may at any time be overturned by new information about mechanisms of action, so that an interaction said to be synergistic one day may on the next be said to be antagonistic, and this with no change whatsoever in the observed data. This does not appear to be a tenable basis for the study of interactions.

Further, as will be suggested below, it should be possible to say from measurements only of effects whether

UNITED THERAPEUTICS CORP., EX1034, page 3
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

different agents interact or not to produce these effects, even when the nature of the agents is unknown, and consequently no information is available as to modes of action.

Clearly, therefore, synergy and antagonism cannot usefully be defined as departures from what is expected from mechanisms of action. In fact, there are many good examples of well-founded mechanistic models satisfactorily accounting for the effects of drug combinations, whether these be synergistic, zero-interactive, or antagonistic (215, 268–270, 539, 540), and none of the investigators concerned made the mistake of defining interaction as that which is unexpected from the model.

If departure from expectation based on mechanisms of action is discarded as a basis for classifying and measuring interactions, there remain only the observed effects of the agents and their combinations, considered without reference to mechanisms. Unlike our understanding of mechanisms of action, such measurements do not change as knowledge changes, although they may become more accurate or more refined in other ways. Further, an approach based on these is just as applicable to agents of which the modes of action are completely unknown as to those that are well understood, and even to agents that are unidentified. On this basis, interaction is defined as being present when the effect of a combination of agents differs from that expected from their individual dose-response curves. That is, a general, empirical model of zero interaction is constructed, based only on observed dose-response relations of the agents, and interaction is defined as departure from the model.

It is therefore useful to consider just what would be required of such an empirical, mechanism-free model, and how its validity could be tested. The requirements are analogous to those of a nonparametric statistical test. There are, for example, some statistical tests (the Mann-Whitney U test, the Kolmogorov-Smirnov test, and so on) that calculate the probability that two sets of values come from populations with the same distribution and, to decide this question, no information is required about what the values represent nor about the real distributions of the populations from which the sets of values came. Analogously, a valid mechanism-free method for examining interactions between agents should be able to show whether or not two (or more) agents interact or not simply on the basis of observed effects. No information should be required about the nature of the agents or their mechanisms of action, nor about whether they are in fact the same or different.

The question then arises as to how the dose-response relations of agents may be used to construct a model giving the expected effect of a combination. Unfortunately, there is considerable confusion on this question, so much so that for the past 30 years and more it has been almost an established custom to begin reviews on synergy with a discussion of the prevailing confused and anarchic state of affairs (39, 42, 193, 209, 480, 508, 513, 514). At least eight different approaches are commonly used. In describing these, the following symbols will be used for combinations of two agents A and B (their extension to any number of agents numbered 1,2,,,,, n will usually be elementary). The combination is termed (d_a,d_b) where d_a and d_b are the doses (or concentrations if appropriate) of A and B, respectively. Effect is treated as a mathematical function E; thus, $E(d_a,d_d)$ or, where an explicit algebraic function can be used, $f(d_{a,b})$ is the effect of the combination. Where E is expressed as a fractional effect (for instance, fractional cell kill or fractional inhibition of an enzyme), then S is the surviving fraction, i.e., $S = 1 - E$. D_a and D_b are the doses of A and B separately that are isoeffective with the combination.

The commonly used approaches for analysing interactions are as follows:

(*a*) Construction of isoboles (iso-effect curves), in which the combination (d_a,d_b) is represented by a point on a graph the axes of which are the dose-axes of the individual agents. It is expected that, if the agents do not interact, the isobole joining the point representing the combination to those on the dose-axes representing doses isoeffective with the combination (D_a and D_b) will be a straight line (39, 40, 42, 44, 47, 182, 183, 200, 247, 333, 335, 338, 510, 547) (fig. 1). For combination of three agents, the isobole is a surface in three dimensions (39, 40, 42, 127, 159) and the zero-interaction isobole is a flat plane.

The equation for the zero interaction line for two agents is

$$\frac{d_a}{D_a} + \frac{d_b}{D_b} = 1 \qquad (1)$$

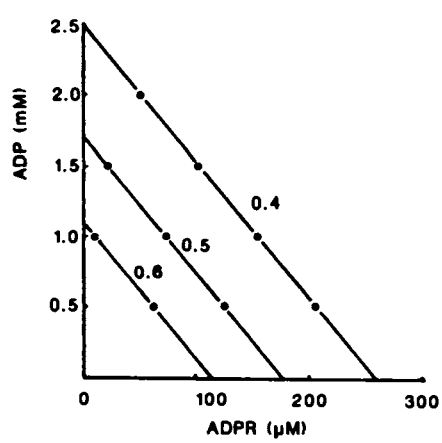

FIG. 1. Isoboles showing zero interaction between adenosine diphosphate ribose (ADPR) and adenosine-5′-diphosphate (ADP) in inhibiting alcohol dehydrogenase (taken from fig. 4 of Yonetani and Theorell (557)). Isoboles are shown for fractional residual activities of 0.4, 0.5, and 0.6. The isoboles joining points representing isoeffective combinations (●) to points on the axes representing isoeffective concentrations of the agents used on their own are all straight lines.

UNITED THERAPEUTICS CORP., EX1034, page 4
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

(and, for n agents, it is

$$\sum_{i=1}^{n} \frac{d_i}{D_i} = 1 \quad (i = 1, 2, \cdots, n) \qquad (1a)$$

For example, in fig. 1, for the combination of 50μM ADPR with 2 mM ADP, which leaves a fractional enzyme activity of 0.4, the isoeffective concentrations of ADPR and ADP are 250 μM and 2.5 mM, respectively. Thus, equation 1 is satisfied, for 50/250 + 2/2.5 = 1, and the isobole for this effect is a straight line.

When agents in combination are more effective than expected from their dose-response curves (synergy), smaller amounts are needed to produce the effect under consideration, i.e., d_a and/or d_b are reduced, while D_a and D_b, being doses of the agents used on their own, are unchanged, so

$$\frac{d_a}{D_a} + \frac{d_b}{D_b} < 1 \qquad (2)$$

(This expression is strictly an inequality, not an equation, but it and similar expressions used here will be termed equations simply for convenience in listing).

Equation 2 defines a concave-up isobole. For example, in fig. 2, for the combination of 74.5 mg/kg of trimethadione with 8.25 mg/kg albutoin, the isoeffective doses are 298 mg/kg and 33 mg/kg, respectively, so the relevant equation is 74.5/298 + 8.25/33 = 0.5.

Conversely, when the agents in combination are less effective than expected (antagonism), d_a and/or d_b must be increased to produce the required effect, so that

$$\frac{d_a}{D_a} + \frac{d_b}{D_b} > 1 \qquad (3)$$

Equation 3 defines a concave-down isobole. For example, in fig. 3, for the combination of 760 mg/kg trimethadione with 175 mg/kg albutoin, the isoeffective doses are 1520 mg/kg and 210 mg/kg, respectively, so the equation is 760/1520 + 175/210 = 1.33.

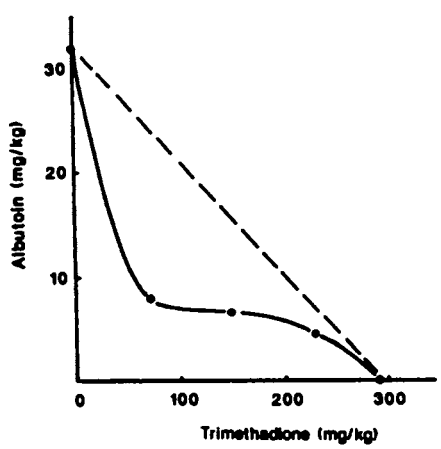

FIG. 2. Isobole showing synergy between trimethadione and albutoin in protecting mice against clonic seizures induced by pentylenetetrazol (redrawn from fig. 3 of Wallin et al. (524). The dashed line indicates the zero interaction isobole, i.e., the locus of all combinations that would have produced this effect if there had been no interaction. The three combinations (●) shown are all to the left and below this line, showing that less of the drugs is needed than expected, i.e., they are synergistic.

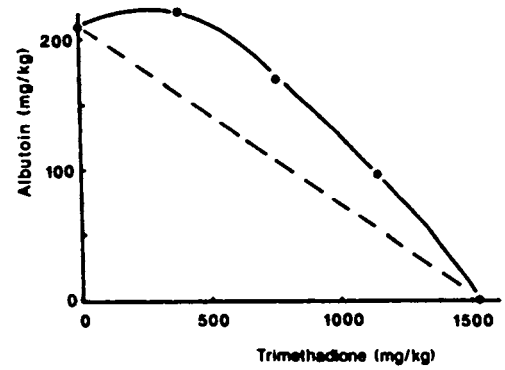

FIG. 3. Isobole showing antagonism between trimethadione and albutoin in protecting mice against electrically induced tonic extensor seizures (redrawn from fig. 3 of Wallin et al. (524)). The three combinations shown are all above and to the right of the zero interaction line, showing that the drugs are less effective in combination than expected.

The sum in equations 1–3 is an interaction index which measures the divergence between the amounts of the agents that are observed to produce a given effect in combination and the amounts that would be expected to do so from their dose-response curves. The use of this index as a measure of interaction originated with Henle (245), its connection with graphic methods was recognised by Frei (185), and with the classic isobole method by Loewe (334,341).

The earliest published isoboles, dating from 1870, are those of Fraser (182,183), for the lethal effects of combinations of physostigmine and atropine, which showed antagonism. There is little evidence of the subsequent use of this method until the publication of a series of papers by Loewe and his colleagues in 1926–7, most of which concerned the lethal effects of combinations of analgesics and barbiturates, which showed antagonism (276–279, 332, 340, 341). One paper from this group (468), on the lethal effects of combinations of strophanthus and digitalis, appears to be the first to illustrate a synergistic isobole. In spite of this small spate of publications, widespread adoption of the isobole method had to wait another 20 years (378, 97, 105, 142, 160). Experimentally determined isoboles have since been published for narcotics (132, 201–204, 292–294, 383, 384, 466, 505a, 506, 507, 523, 541, 556), anti-convulsants and combinations of these with convulsants (105, 336, 337, 339, 356, 524), α- and β-adrenoceptor agonists (238), cytotoxic drugs and antimetabolites (35, 160, 216, 256, 260a, 267, 398, 437), antibiotics (172, 239, 287, 378, 395, 501, 559), antiviral agents (25, 127, 133, 274, 291, 368, 457, 464a), anticoccidial agents (186), antitoxoplasmal agents (54), antileishmanial agents (354), antitrypanosomal drugs (512), antimalarial drugs (104, 309, 444), immunosuppressive agents (49), insecticides (113, 201), agents inducing acute shock (425) and for combinations of physical stimuli such as noise and vibration (59, 244). The

UNITED THERAPEUTICS CORP., EX1034, page 5
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

papers by Tverskoy et al. (505a, 506, 507) appear to be the first to show isoboles for drug effects in man.

(*b*) Summation of effects, in which the effect of a zero interactive combination is expected to be the sum of the effects of its constituents, i.e., $E(d_a d_b) = E(d_a) + E(d_b)$ (1, 63, 95, 96, 118, 135, 150, 159, 196, 199a, 212, 230, 234, 237, 262, 288, 345). Methods based on analysis of variance (71a, 83, 179, 217, 234, 281a, 290, 308, 316, 317, 319, 432, 438, 449, 450, 470, 490, 549) come under this heading, for linearity of response is a basic assumption of this approach, in which interaction is *defined* as departure from summation of effects.

(*c*) Multiplication of surviving fractions, in which fractional survival after treatment with a zero-interactive combination is expected to be the product of the fractional survivals after treatment with each of of its constituents, i.e., $S(d_a d_b), = S(d_a). S(d_{bb})$ (29, 36, 70, 80, 108, 140, 151, 164a, 178, 286, 295, 296, 352, 353, 441, 469, 513, 522, 538). An equivalent method, used particularly by radiobiologists, involves measuring the effect of a fixed dose of one agent on the survival curve of the other, zero interaction being deemed present if the curve is shifted downwards by a fixed distance on a logarithmic scale, while changes in slope are held to indicate an interaction (145, 227, 508).

(*d*) A more complicated version of the isobole method in which zero interaction is represented by an envelope between two limiting isoboles, at least one of which is curved, rather than by a single straight line (137, 190, 246, 295, 370, 371, 387, 415, 453, 454, 479, 480, 485, 486, 492, 494, 504, 553).

(*e*) Measurement of the effect of a fixed dose of one agent on the dose-response curve of the other, zero interaction being deemed to be present if the dose-response curve is shifted horizontally by a fixed distance on a linear scale (149, 409–412). (Commonly a logarithmic dose scale is used, in which case the original and the shifted dose-response curves are not parallel but converge at higher doses).

(*f*) Calculation of the effect of a zero-interactive combination from the law of mass action (110–113, 231, 258, 304, 442, 499, 518).

(*g*) Comparison of the effect of a combination with that of its constituents, synergy being deemed present if the former exceeds each of the latter, i.e., $E(d_a d_b) > E(d_a)$ and $E(d_b)$ (170, 222, 263, 392, 422, 430, 463). This approach owes much to Gaddum (189), who argued that, if the effect of a combination exceeds those of its constituents, they must necessarily be helping each other, and that only combinations with effects less than that of one or more constituents should be termed antagonistic. Thus, Gaddum drew isoboles for various interactions in the manner of fig. 4.

(*h*) The last approach to be noted here, and the one that is by far the most often used, is that in which explicit criteria are conspicuous by their absence. Here,

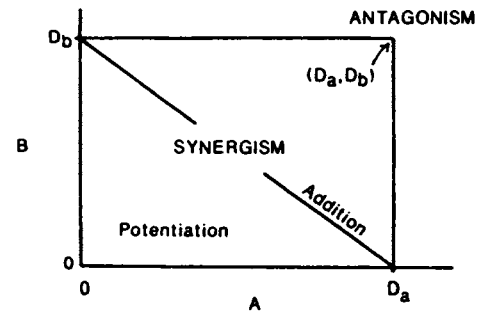

FIG. 4. Isobolar diagram of Gaddum (189). Synergy is here said to be present when the effect of a combination exceeds that of either of its constituents and thus all combinations in the rectangle shown are said to be synergistic. Antagonism is said to be present only when the combination is less effective than any of its constituents (see text for comments). Synergy in the sense used in this review (cf. fig. 2) is termed potentiation by Gaddum, and the zero interaction isobole is said to show "addition."

authors claim to have demonstrated synergy without specifying any method or criterion at all, apparently assuming that the conclusion is self-evident, or possibly being unaware that several different methods are used and that there is a problem of selection. Some recent examples are refs. 26, 28, 72, 74, 84, 129, 164, 311, 306a, 326, 364, 455a, 493, 529, and 563.

These methods are of varied status. Methods (*a*) and (*g*) are strictly mechanism-independent and are thus based on truly empirical models. The remaining methods (excluding (*h*), the "no method" approach) are almost universally treated as if they were empirical, mechanism-free methods but, as will be shown below, they all depend on assumptions about the shapes of the agents' dose-response curves and thus on their mechanisms of action. Most investigators appear to be unaware of these underlying assumptions.

This list is by no means comprehensive. Some less well-defined methods are mentioned by Goldin and Mantel (209), and new methods of little or no validity continue to appear with regrettable frequency, serving only to add to the confusion.

It is not surprising that these different methods may give different results when applied to the same set of data, so that a combination may appear synergistic according to one method and antagonistic according to another. For example, if each constituent of a two-agent combination alone inhibited some activity by 50% and a combination of both inhibited it by 90%, use of the summation method would indicate antagonism (100% inhibition expected) while use of the multiplicative method would indicate synergy (75% inhibition expected).

The coexistence of several methods, some with energetic advocates, not unnaturally gives rise to the belief that all methods are equally valid, or that their validity is inherently untestable or that the matter is simply one of semantics. Thus, choice of method is often asserted to be a matter of personal preference, depending perhaps

on the nature of the problem but more often on the investigator's familiarity with the method or simply on statistical convenience (485, 525).

One reflection of the prevailing uncertainty is that some reviewers merely list a variety of methods without clearly specifying the circumstances, if any, in which they are appropriate (79, 144, 365, 485). Another is the practice of applying two different methods to the same data, on the supposition that, even if the methods are unsure, the conclusion is more likely to be correct if both methods agree as to the type of interaction present (231, 232, 260a, 281, 415, 441, 464a, 516).

In fact, there is a generally valid method, the construction of isoboles (method (*a*)) but, until recently, its general validity was unproven. Fraser, the originator of the method (182, 183), gave no rationale for the construction, nor was there any need for this in the experiments he described. These concerned the interaction between physostigmine and atropine in causing death and, as atropine was found to counter the lethal effects of physostigmine, there was no difficulty in deciding that the interaction was one of antagonism. Fraser thus invented isoboles simply as an elegant method for displaying data graphically for combinations of two or three variables. (the interval between the two injections was the third variable).

Loewe (333–335, 338, 341), who developed the method further and applied it to synergistic combinations, gave as its rationale the fact that a straight line isobole would always result in the case of a sham "combination" of an agent with itself and, intuitively, this should apply also to combinations of different agents if they had similar modes of action and similar dose-response curves. However, Loewe rightly felt that such intuitive reasoning could not very well be generalised without further justification to include combinations of agents with different mechanisms of action and dissimilar dose-response curves, and he asserted that in such cases the isobole for zero interaction (additivism) would be curved. No convincing reasons for this assertion was put forward but this, and similar assertions by other authors, have been the source of some subsequent confusion (see section VIII below).

It will be shown here that the isobole method is a generally valid procedure for analysing interactions between agents irrespective of their mechanisms of action or the nature of their dose-response relations. Each of the other methods except method (*g*) (and, of course, method (*h*)) is valid in particular circumstances, and the isobole method and its algebraic representation will be used to show what these circumstances are. Method (*g*) is soon disposed of by testing it on a sham "combination" of two doses of one and the same agent. For all agents in the dose-range where effect increases with dose, the effect of this "combination" must exceed the effect of each of its "constituents." Thus, the conclusion according to method (*g*) is that the "combination" is synergistic. This is clearly incorrect, for the effect can only be precisely that which is expected from the dose-response curve of the agent. Some may find more appealing the down-to-earth argument of Smith (464) If two men, working separately, can each cut down 10 trees in a day but, working together, they cut down 15, then they must somehow be working against each other (perhaps by getting in each other's way), not helping each other. Yet the effect of this "combination" exceeds the effect of each of its "constituents" and, according to criterion (*g*) and Gaddum's isoboles, they would be supposed to be acting synergistically.

III. Terminology and Definitions

One of the causes of confusion in the literature on drug interactions is the profusion of terms and lack of agreement on their definitions. For example, Fedelli et al. (171) and Steel (479) each define 11 different types of interactions, but each uses a different terminology and there appears to be little if any correspondence between their classifications.

There is no need, in the present context, to define more than three classes of interactions: zero interaction, in which the effect of a combination is that expected from the dose-response curves of the agents; synergy, in which the effect is greater than expected; and antagonism, in which it is less. A commonly used synonym for zero interaction is additivism (or addition), but this term will generally be avoided here as it has acquired several different meanings in the literature. Many other terms are used (potentiation, augmentation, enhancement, etc), but these again are either defined differently by different authors or, more often, are used without definition, so they will also be avoided in this review.

IV. Parameters for Assessing Interactions

The key to finding a generally valid method for assessing interactions between agents is to decide on what parameters of a combination could be used for this purpose. If the method of assessment is to be generally applicable, the parameters chosen must be capable of specification and measurement irrespective of the nature of the agents' dose-response relations. For instance, parameters such as slope of the dose-response curve, which apply only to curves that are linear (or linearisable) over the relevant part of the dose-range, or degree of sigmoidicity, which apply only to sigmoid curves, are not general in this sense. There are four completely general parameters for any combination of A and B. Two are related to the individual constituents, i.e., (i) the doses of the constituents, d_a and d_b, and (ii) the effects of the constituents, $E(d_a)$ and $E(d_b)$; and two are related to the combination, i.e., (iii) the effect of the combination, $E(d_a, d_b)$, and (iv) the doses of the individual agents isoeffective with the combination, D_a and D_b. (In the case of agents that, on their own, do not in any dose

produce the effect of the combination, the appropriate value of D_a or D_b (or both) is set at infinity and this gives rise to no difficulty in any of the procedures described below). The only case in which any question arises about the values of these parameters is that of agents with nonmonotonic dose-response curves, where there may be more than one value for D_a and/or D_b, but such cases do not generally give rise to any difficulty in practice, as the correct value to choose is usually fairly obvious from inspection of the curves.

Now the question as to whether putting the constituents together to form a combination leads to an interaction or not cannot be answered by considering only relations between constituent-related parameters (i) and (ii) on the one hand or only those between combination-related parameters (iii) and (iv) on the other. A relation is required between constituent-related parameters and combination-related parameters. Intuitively, it would appear that the obvious connection to seek is one between effects, i.e., between (ii) and (iii). Some connections that have been proposed have been mentioned above, for instance that $E(d_a,d_b) = E(d_a) + E(d_b)$ or that $S(d_a,d_b) = S(d_a)\cdot S(d_b)$. However, as such solutions generally yield different answers with the same set of data, they cannot all be generally valid.

Now there is one sort of combination the effect of which must always be precisely that expected from the dose-response curves of its agents, irrespective of their shapes, and that is the sham "combination" of various doses of one and the same agent. If the sham combination test is applied, for instance, to an agent with a linear dose-response curve, the summation method would always give the correct answer, for here, if d_1 and d_2 are two doses of the same agent, then the curve shows that $E(d_1,d_2) = E(d_1) + E(d_2)$. However, the multiplication method would generally not give the correct result (see section VI B 1). On the other hand, if this test is applied to an agent with an exponential dose-response curve, the multiplication method would give the correct result (see section VI B 2) while the summation method generally would not. Neither the summation nor the multiplication method would generally yield the correct answer if applied to sham combinations of an agent with a dose-response curve that was sigmoid or exponential with a shoulder. Thus, methods that attempt to calculate the effect of a zero-interactive combination directly from the effects of its constituents apply only in restricted circumstances, each such method being valid only for agents with particular types of dose-response curves. Further, any such method can be used only when all the agents in a combination have dose-response curves of the same general type. For example, neither the summation nor the multiplication method could be applied in general to combinations in which the dose-response curve of one agent was linear while that of the other was exponential.

Evidently, seeking a connection between the effect of the combination and those of its constituents cannot lead to a generally valid solution. The alternative is to seek a connection between (i) the doses of the constituents, d_a and d_b and (iv) the isoeffective doses of the individual agents, D_a and D_b. The nature of this connection may be shown by examining the properties of the sham "combination" described above.

V. Isobole Method

A. General Validation

The isobole method depends on the fact that, if a combination (d_a,d_b) is represented by a point in a graph the axes of which represent doses of A and B respectively, then the point lies on the straight line joining D_a and D_b (and thus d_a, d_b, D_a, and D_b satisfy equation 1) if and only if the combination is zero-interactive (fig. 1).

The isobole method is well established and is widely used in many fields. However, until recently, it suffered from the lack of a formal proof of its general validity. In addition, there is the difficulty created by Loewe's doubts as to its general validity, his assertion (334, 335) that, if used for combinations of agents with dissimilar dose-response curves, it would generate curved isoboles even if there were no interaction and his belief that, for combinations of two such agents, there were two possible forms for each isobole. Fortunately, most investigators seem to have ignored (or possibly have been unaware of) these doubts, and have quite correctly used the isobole method irrespective of the similarity or dissimilarity of the dose-response curves of the agents in question.

Nevertheless, the doubts have persisted and have caused considerable confusion, and it is clearly essential for the future well-being of this field of study that, if the isobole method is indeed generally valid, a convincing proof of this be presented. The proof is given here for combinations of two agents, but it is readily generalised for combinations of any number (44).

The proof depends on constructing a sham combination that mimics the real combination in the criteria listed above, i.e., it should have the same values for d_a, d_b, D_a, and D_b. It must mimic the real combination in effect, otherwise the exercise would be pointless, and it must be indisputably zero-interactive. This last aim is achieved simply by using only one agent (and dilutions of that agent) to make up the sham combination. The effect of such a combination can only be that expected from the dose-response curve of the agent selected, so it is by construction zero-interactive. If a real combination cannot be distinguished from a zero-interactive sham combination by the criteria discussed above, it must also be zero-interactive according to these criteria. Either A or B could be used to make the sham combination. We shall select A. To construct a sham agent to mimic B, A is diluted (D_b/D_a)-fold. This material will be called A'. Clearly, the dose of A' isoeffective with D_a (and D_b) is D_b units, because this contains $(D_a/D_b)D_b$ units of A, i.e.

UNITED THERAPEUTICS CORP., EX1034, page 8
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

D_a units of A. Therefore, A' mimics B in the only relevant respects, i.e., the magnitude of D_b and its effect.

The sham combination contains d_a units of A and d_b units of A', so it has the same values for d_a and d_b as the real combination. Whether the real combination is zero-interactive or not depends on whether it and the sham combination have equal effects. Now the sham combination is, in fact, d_a of A plus $(D_a/D_b)d_b$ of A. If this and the real combination are isoeffective, then

$$E(d_a \text{ of } A + d_b \text{ of } B)$$
$$= E\left(d_a \text{ of } A + \frac{D_a}{D_b} \cdot d_b \text{ of } A\right) = E(D_a \text{ of } A)$$

Therefore, for agents with monotonic dose-response curves,

$$d_a + \frac{D_a}{D_b} \cdot d_b = D_a$$

Dividing throughout by D_a gives equation 1.

Thus, the real and sham combinations cannot be distinguished from each other in any relevant respect (d_a, d_b, D_a, D_b, or effect) and, as the sham combination is zero-interactive, so also must be the real one.

Equation 1, which describes a straight-line isobole, gives the relation sought between d_a, d_b, D_a, and D_b for a zero-interactive combination. Its derivation took no account, either explicitly or implicitly, of the shapes of the dose-response curves of the agents or of their mechanisms of action, and thus it is valid irrespective of the shapes of these curves and of whether they are similar or dissimilar, and irrespective of their mechanisms of action.

Now suppose that the true combination (d_a of A and d_b of B), which is isoeffective with Da, is more effective than the zero-interactive sham combination (d_a of A and $(D_a/D_b).d_b$ of A), i.e., it shows synergy. Then

$$E\left(d_a \text{ of } A + \frac{D_a}{D_b} \cdot d_b \text{ of } A\right)$$
$$< E(d_a \text{ of } A + d_b \text{ of } B) = E(D_a)$$

Thus,

$$d_a + \frac{D_a}{D_b} \cdot d_b < D_a$$

and dividing throughout by D_a gives equation 2.

Conversely, if the true combination is less effective than the zero-interactive sham combination, i.e., if it shows antagonism, then entirely similar reasoning gives equation 3.

B. Remarks on the Isobole Method

1. Data required for assessing interactions. It is quite unnecessary to have complete dose-response curves of all the agents in a combination in order to detect and categorize an interaction (this is sometimes incorrectly claimed to be a drawback of the isobole method (112, 362). In many cases, only limited determinations may be enough to show that the isobole must of necessity be concave-up or concave-down (42). For example, Archer et al. (12) found that a combination of 30 ng PAF-acether and 0.5 μg PGE_2 injected intradermally into human skin produced a wheal of about 70 μl volume (fig. 5). Wheals of this size were not produced by the maximum doses of either agent used alone, 60 ng of PAF-acether or 1 μg PGE_2, so the interaction index for the combination is necessarily less than 30/60 + 0.5/1.0, i.e. <1.0, and the isobole for the effect is necessarily concave-up, indicating synergy.

Thus, although the values of D_a and D_b in equation 2 were not determined, the combination was undoubtedly synergistic.

In two sets of circumstances, unequivocal decisions may be made with even less information about the dose-response curves, as follows.

(*a*) If a combination is less effective than one or more of its constituents, then no further information is needed in order to conclude that antagonism is present. For example, fig. 6 shows that a combination of 0.5% v/v halothane in inspired air with 306 mg/kg morphine prevented the increase in heart rate in response to a noxious stimulus in rats (293). However, the same effect could be produced by 5.9 mg/kg morphine alone, so that the the 306 mg/kg in the combination must have been very much less effective than 306 mg/kg morphine on its own. Here, the combination (d_a, d_b) is (0.5, 306) and the isoeffective dose of morphine (D_b) is 5.9. Thus, d_b/D_b = 306/5.9 = 51.9. Clearly, even without knowing anything about the isoeffective dose of halothane, it is certain that the interaction index for this combination greatly exceeds unity, and the isobole for the effect is markedly concave-

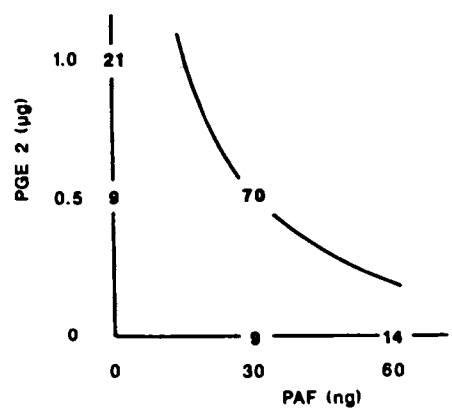

FIG. 5. Isobole showing the interaction between PAF-acether and PGE_2 in inducing wheal formation in human skin (data from table 2 of Archer et al. (12)). A 70 μl wheal is produced by a combination of 30 ng PAF and 0.5 μg PGE_2. However, a wheal this size is not produced by double these amounts of the agents used on their own. Thus, the isobole for combinations producing a 70 μl wheal meets the individual dose-axes at higher doses than these (if it meets them at all), and therefore it must be concave-up. Accordingly, it may be possible to classify a combination as synergistic even when the isoeffective doses of the individual agents are unknown.

UNITED THERAPEUTICS CORP., EX1034, page 9
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

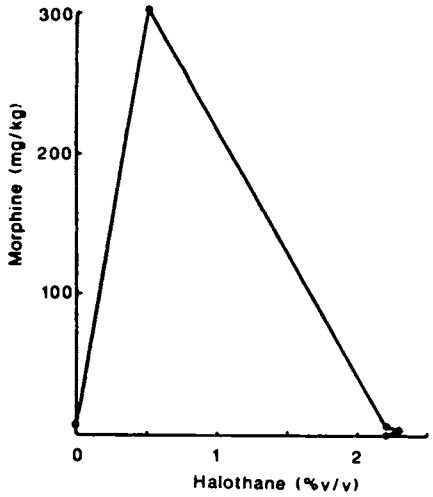

FIG. 6. Isobole showing marked antagonism between halothane and morphine in abolishing the tachycardia in response to noxious stimuli in rats (redrawn from fig. 5 of Kissin et al. (293)). A combination of 0.5% v/v halothane with 306 mg/kg morphine has the same effect as 5.9 mg/kg morphine on its own. Therefore, from these two determinations alone, and without any information about the dose response curve for halothane, it is certain that marked antagonism is present.

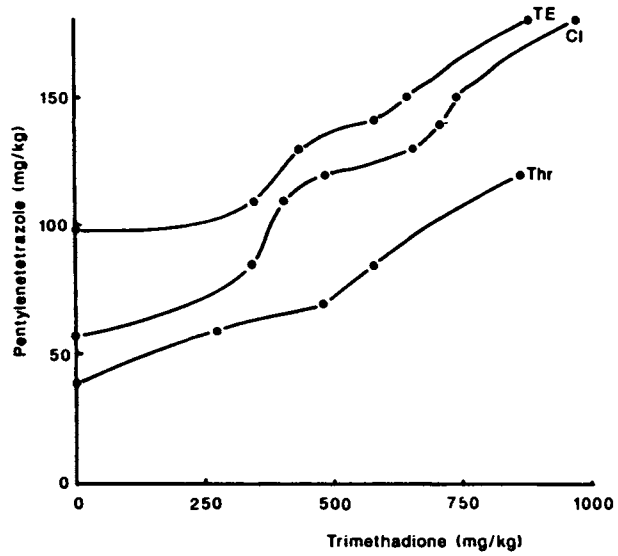

FIG. 7. Isoboles for heterergic antagonism (redrawn from fig. 1 of Loewe et al. (339)). Pentylenetetrazole is a convulsant in mice and, depending on the dose, produces threshold level convulsions (Thr), general clonic seizures (Cl), or tonic extensor seizures (TE). Trimethadione does not produce these effects but increases the doses of the convulsant required to produce them, i.e., it is an antagonist. Thus, the isoboles for these effects do not meet the trimethadione dose-axis at any dosage, and are deflected away from this axis.

down with respect to the origin, indicating marked antagonism.

(*b*) If one of the agents does not in any dose produce the effect of the combination and the effect of the combination differs from that of the active constituent, then an interaction must be present. Such combinations were termed "heterergic" by Loewe (335). If, say, the active agent is *B* and the effect of the combination exceeds that of the active constituent d_b, then D_b must exceed d_b, so $d_b/D_b < 1$. But, as *A* is inactive, D_a is notionally infinite, so d_a/D_a may be taken to be zero. Therefore the interaction index is less than 1 and synergy is present. On the other hand, if the effect of the combination is less than that of the active constituent d_b, then $D_b < d_b$, so $d_b/D_b > 1$, the interaction index must exceed 1 and antagonism is present. These points may be illustrated by fig. 7, which shows isoboles for combinations of trimethadione and pentylenetetrazole in respect of production of threshold level convulsions, general clonic seizures, and tonic extensor seizures (339). These effects are produced by pentylenetetrazole on its own, but not by trimethadione, so the combinations are heterergic. For example, with the combination of 875 mg/kg trimethadione and 180 mg/kg pentylene-tetrazole, which produces tonic seizures, (d_a,d_b) = (875,180) and D_b, the isoeffective dose of pentylenetetrazole, is 100 mg/kg. Thus, d_b/D_b = 180/100 = 1.8, so the interaction index must exceed 1 and antagonism is present. The isoboles are displaced away from the trimethadione axis, so they are concave-down with respect to the origin.

A special case of this class is that in which none of the agents on its own can produce the effect of the combination. Loewe (335) termed this "coalitive interaction," and it is illustrated by fig. 23, which shows the prolongation of survival in mice with L1210 leukaemia given combinations of carminomycin and cyclophosphamide (24). Prolongation of 150% or more in survival is not produced by any dose of either agent used alone, but it is produced by combinations. Thus the isoboles for these levels of effect are closed curves that touch neither dose axis. Both D_a and D_b are notionally infinite so that the interaction index is zero, i.e., this represents the limiting case of synergy.

It is also sometimes possible to draw tentative conclusions about the expected effect of a combination simply from the effects of its constituents and without specific information about the dose-response curves of the agents when it is permissible to assume the shapes of these curves. For example, dose-response curves generally approximate to linearity in the low dose/low effect region and, in these circumstances, it is reasonable to expect that, in the absence of interaction, the effect of a combination will be the sum of the effects of its constituents (see section VI B 1). For instance, for most human carcinogens, we are in the region in which linearity of response is a reasonable assumption (125, 131, 219, 220, 401), so that the observation that, for many carcinogen combinations, the observed cancer incidence considerably exceeds the sum of incidences attributable to the individual carcinogens (58, 418, 435, 446–448) suggests that they act synergistically. Conclusions based on such evidence should be regarded as provisional until sufficient information about the dose-response curves is obtained.

When no adequate assumptions about dose-response

UNITED THERAPEUTICS CORP., EX1034, page 10
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

curves can be made, it is quite unjustifiable to guess at the expected effect of a combination solely on the basis of the effects of the doses of the individual constituents. However, this is unfortunately a common practice; many authors simply assume arbitrarily that the expected effect of a combination is the sum of the effects of its constituents, or the product of these effects, and draw conclusions accordingly as to the supposed nature of the interaction. Such assumptions underlie the commonly used 2 × 2 experimental design in which agents are used at single doses alone and in the same doses in the combination (1, 6, 95, 136, 164, 179, 282, 493). It will be evident from the preceding discussion that such experimental arrangements are incapable of detecting synergy, zero interaction or moderate antagonism. They are capable only of detecting that level of antagonism in which the combination is less effective than one or more of its constituents. This limitation holds even when the individual constituents have little or no effect while the combination has a marked effect, for a sham (zero interactive) combination of one and the same agent might behave in this way if its dose-response curve was initially shallow (or had a threshold) and then rose steeply.

The so-called "killing-curve" method of bacteriologists also reflects the idea that interactions may be assessed in the absence of any information about dose-response curves. Here, it is asserted that synergy is present if bacterial growth with a combination of antibiotics is less than that with the more effective constituent alone to an extent that different workers variously set at 10-fold (187), 100-fold (224, 385, 400, 561), 1,000-fold (537), 10,000-fold (501), and so on. These quite arbitrary and variable levels serve only to show that, in the absence of information about the dose-response curves for the effects of the antibiotics on bacterial growth, criteria such as these are without basis. In fact, when the dose-response curve is sufficiently steep, even the most stringent of these criteria may easily be met by a sham combination of an antibiotic with itself (40).

2. Isoboles are not necessarily consistent or similar. While it is commonly found that the isobole for a particular level of effect shows one type of interaction consistently throughout its course (i.e., all combinations showing a particular effect being synergistic, or antagonistic or zero interactive as in figs. 1 to 3), this is by no means always the case.

Isoboles may cross the zero-interactive line so that some combinations with a specified effect are synergistic and others antagonistic (25, 172, 202, 287, 384) (fig. 8). One of the advantages of multi-agent combinations appears to be a reduction in this inconsistency. Berenbaum et al. (50) found, in testing combinations of two or three antibiotics against *Peudomonas maltophilia*, that 30% of the isoboles for combinations of two antibiotics were inconsistent whereas, for triple combinations, this proportion was reduced to 10% and, moreover, the degree of inconsistency remaining was negligible.

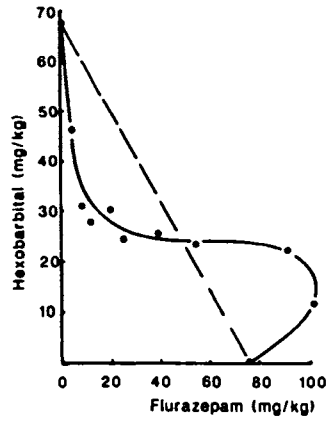

FIG. 8. Isobole for anaesthetic effects of fluorazepam and hexobarbital (redrawn from fig. 3 of Norberg and Wahlström (384)). The isobole is markedly inconsistent, with synergy in one region, antagonism in another, and zero interaction at one point.

Further, for any particular set of agents, it is often the case that isoboles for different types of effect or for different levels of the same effect behave dissimilarly (105, 202, 291, 293, 336, 337, 398, 506, 507, 524). Compare, for instance, figs. 2 and 3, which show isoboles for two different effects for the same set of agents, one showing synergy and the other antagonism. Therefore, the conclusion as to whether a combination shows synergy, zero interaction, or antagonism applies specifically to that combination and such conclusions should not be generalised to untested combinations.

3. Statistical examination of isoboles. Several authors have considered how isoboles might be evaluated statistically. A common approach is to plot the isobole with confidence limits (132, 200–205, 239, 395, 466, 541) or standard errors (105, 383, 384, 523, 524). Kissin and his colleagues (292–294, 506, 507) use a propagation of error method to assess deviations from the linear isobole. More elaborate methods have been devised by Scaf (439), Wolf and Unkelbach (547), and Carter *et al.* (87), but each of these appears to depend on assumptions about the shapes of the dose-response curves, for example, that the curves for the different agents in the combination are similar to each other and linear with log dose (439), or that they fit a probit or logistic model (87, 547).

Plotting an isobole often involves examining the effects of several combinations, and appropriate statistical tests may be applied to each combination separately. It has been argued (87) that such multiple testing artificially increases the likelihood of finding a significant deviation from zero interaction, but it should be emphasised, as explained above, that the nature and extent of an interaction is a property of the individual combination, and testing each one in a multi-combination experiment is not an invalid procedure.

Nevertheless, it is worth considering whether statistical tests may be applied to a set of combinations to

determine the overall nature of an interaction. One such attempt has been made by Tsai *et al.* (504). These workers located in an isobologram 5 to 10 combinations that produced 50% cell survival, determined the proportion falling below and to the left of the additivity envelope (an unfortunate choice of method—see section VII below), and used a binomial test to examine the hypothesis that this proportion was significantly less than 0.5. A more sophisticated approach was made by Carter *et al.* (87), who used the maximum likelihood method to fit experimental results to a logistic model. This made it possible to test the model for adequate fit to the data and to calculate the significance of the deviation from zero of the interaction index. Such overall methods may have a use over and above that of statistical tests on individual combinations, especially when none of these deviates significantly from zero interaction, yet a consistent minor interaction, possibly of clinical importance (see section IX A), is present. Overall tests will, however, always come up against the difficulty that interactions are not necessarily consistent, so that a measurement involving all the combinations tested may indicate absence of an overall interaction, even when one is obviously present (fig. 8).

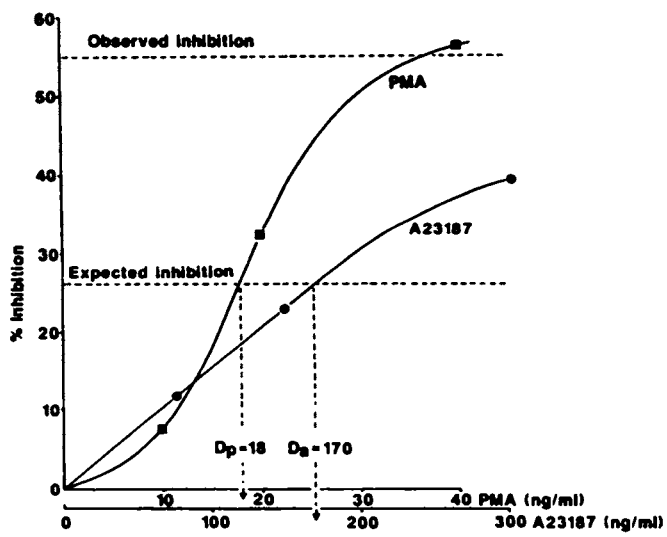

FIG. 9. Calculation of the expected (zero-interactive) inhibitory effect of a combination of 10 ng/ml phorbol myristate acetate (PMA) and 75 ng/ml calcium ionophore A23187 on proteoglycan synthesis in articular chondrocytes. Concentration-inhibition curves are drawn from fig. 3 of Bouakka et al. (63). Given these curves, the object of the procedure is to find concentrations of PMA and A23187 that would be isoeffective with the combination if it were zero interactive (these concentrations are D_p and D_a, respectively). Thus, D_p and D_a must be isoeffective with each other (and are thus indicated by the intersections of the two concentration-response curves with the same horizontal line) and they must satisfy equation 1, i.e., $10/D_p + 75/D_a = 1$. The required values are indicated by the horizontal line intersecting the curves at $D_p = 18$ and $D_a = 170$, for $10/18 + 75/170 = 1.0$. These drug levels would each produce 26% inhibition, so this is the effect of the combination to be expected from the concentration-response curves of the agents. The observed effect was 55% inhibition, so the combination was synergistic.

VI. Calculation of the Expected Effect of a Combination

A. The General Case.

The construction of linear isoboles is, in effect, an answer to the question: if there is no interaction, what combinations are expected to produce a given effect? The complementary question which we now consider is: if there is no interaction, what is the expected effect of a given combination? The first question relates to decrease or increase in the required quantities of agents that might result from using them in combination, while the second relates to increase or decrease in effect. Both questions require answers, as they give different sorts of information. When dose-response curves are steep, a relatively small divergence from the linear isobole may entail a large difference in effect and, conversely, when the curves are shallow, a large divergence from the linear isobole may indicate only a small difference in effect.

In certain cases, when all the agents in a zero-interactive combination have rather simple dose-response curves, it is possible to derive from equation 1 explicit algebraic expressions for the effect of the combination, as will be shown below. However, equation 1 enables us to calculate this expected effect whether the dose-response curves happen to fit simple algebraic functions or not. The method is to find a graphical solution to the equation, i.e., values of D_a and D_b that, with any particular combination (d_a,d_b), satisfy the equation.

The procedure is straightforward. D_a, D_b, and the combination (d_a,d_b) are isoeffective. Therefore, on a graph of the dose-response curves for A and B, the horizontal line at the level of the required effect $E(d_a,d_b)$ intersects the two dose-response curves at points corresponding to D_a and D_b. It is therefore simply a matter of finding which isoeffective doses of A and B read off this graph satisfy equation 1. The horizontal line locating these values indicates the effect of the combination if there is no interaction.

The method will be illustrated in fig. 9 with the data of Bouakka et al. (63) for the inhibitory effect of phorbol myristate acetate, calcium ionophore A23187, and a combination of these on proteoglycan synthesis in articular chondrocytes. For a combination of 10 ng/ml of the former and 75 ng/ml of the latter, the required isoeffective values are 18 and 170 ng/ml, respectively. Thus, the expected effect of the combination would be that of either of these, i.e., 26% inhibition. The observed effect was 55% inhibition, showing that the combination was synergistic. Fig. 10 shows that the isoeffect curve for the expected effect of this combination is linear, whereas the curve for the observed effect is concave-up, indicating synergy.

This method can be applied with no increase in difficulty to combinations of any number of agents. Other examples for combinations of two or three agents are given elsewhere (44, 47) and an example for a combina-

UNITED THERAPEUTICS CORP., EX1034, page 12
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

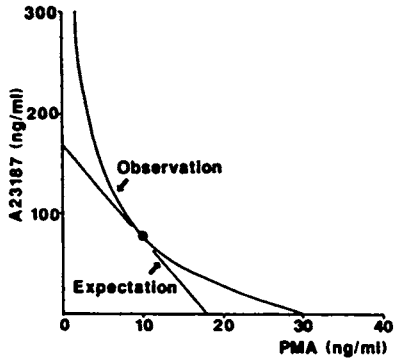

FIG. 10. The isobole for the observed effect of the combination in fig. 9 (55% inhibition) is drawn through the point representing the combination and its ends on the individual concentration axes, at 30 ng/ml PMA and >300 ng/ml A23187, are located from the concentration-response curves in fig. 9. The isobole for the observed effect is therefore concave-up and the interaction index for the combination is less than 10/30 + 75/300, i.e., less than 0.58, indicating synergy. With concentrations of the two agents that have the expected effect of the combination (26% inhibition), i.e., 18 ng/ml PMA and 170 ng/ml A23187, a linear isobole is produced.

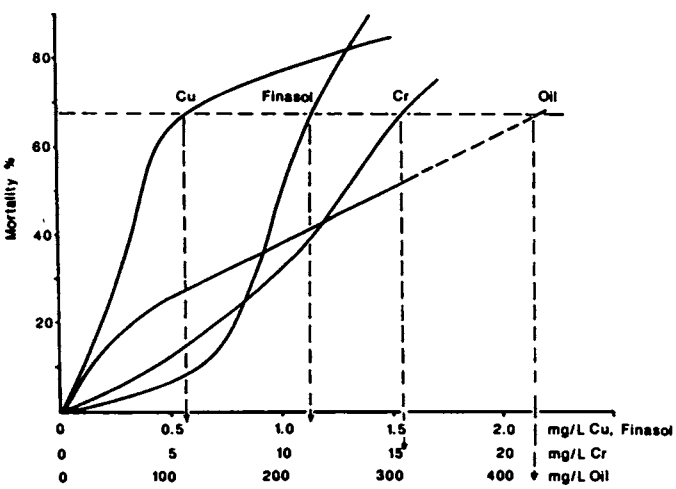

FIG. 11. Calculation of the expected effect of a combination of 0.168 mg/l Cu, 3.873 mg/l Cr, 0.282 mg/l Finasol (an oil dispersant), and 89.34 mg/l crude oil on *Artemia salina* (data provided by Dr. G. Verriopoulos). The procedure is similar to that in fig. 10. A horizontal line is found indicating concentrations of the four agents that (*a*) are isoeffective and (*b*) satisfy equation 1(a). These are 0.57 mg/l Cu, 15.5 mg/l Cr, 1.135 mg/l Finasol, and 430 mg/l oil, for 0.168/0.57 + 3.875/15.5 + 0.282/1.135 + 89.34/430 = 1.00. Each of these produces 67.5% mortality, but the observed mortality was only 20%. Thus the combination showed antagonism.

tion of four agents, each with a different type of dose-response curve, is illustrated in fig. 11. The method is equally applicable in cases in which an explicit algebraic solution is also available. It should be pointed out that the natures of the dose-scales are irrelevant in using this method, but the effect scale must be the same for all agents. In principle, the method could be used with tabulated results, with appropriate interpolation, although a graphical procedure is more convenient. The dose-response curves in these examples have been fitted by eye, but a more objective nonparametric procedure,

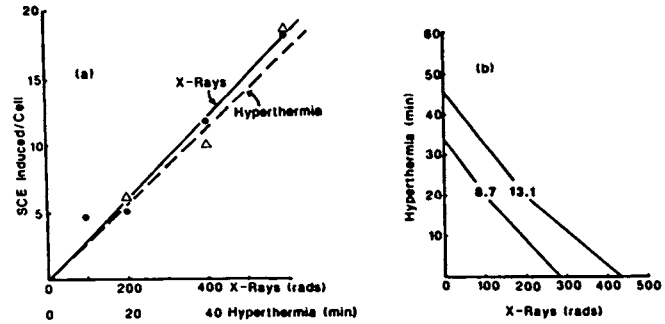

FIG. 12. Use of the summation criterion for interaction. (*a*) Dose-response curves for production of sister-chromatid exchanges (SCE) in Chinese hamster cells by X-rays and hyperthermia (minutes at 44°C) (redrawn from fig. 1 of Livingston and Dethlefsen (329)). Both curves are approximately linear. (*b*) Combinations of 100 and 200 rads X-rays with 20 min hyperthermia were found to induce 8.7 and 13.1 SCE/cell, respectively. The doses of the agents that on their own produced these effects are obtained from the curves in (*a*). The isoboles show slight synergy.

such as the method of monotone spline smoothing of Kelly and Rice (285), would be preferable for regular use.

B. Particular Cases

If the agents in a combination have dose-response curves that are described by explicit algebraic functions, equation 1 may be used to derive an expression for the effect of the combination, as follows.

Rearrangement of equation 1 gives

$$D_a\left[1 - \frac{d_b}{D_b}\right] - d_a = 0 \tag{4}$$

Let the functions describing the dose-response curves for A and B respectively be f_a and f_b. Then, as D_a and D_b are isoeffective,

$$f_a(D_a) = f_b(D_b).$$

If the functions are monotonic,

$$f_b^{-1}f_a(D_a) = f_b^{-1}f_b(D_b) = D_b$$

(For those unfamiliar with this algebraic shorthand, f_a is the function indicating the effect $E(D_a)$ of dose D_a and f_a^{-1} is the inverse function indicating the dose D_a that produces effect $E(D_a)$. $f_b^{-1}f_a$ is the compound function indicating the dose of 8 that produces effect $E(D_a)$ of dose D_a of A. For example, in figure 9, suppose f_a the function indicating the effect of A23187 and f_b that giving the effect of PMA. If the effect under consideration is 26% inhibition, then f_a^{-1} (26%) = 170 ng/ml, which is the concentration of A23187 producing that effect, and $f_b^{-1}f_a$ (170) = 18 ng/ml, which is the concentration of PMA producing the same effect).

Substitution of this expression for D_b into equation 4 gives

$$D_a\left[1 - \frac{d_b}{f_b^{-1}f_a(D_a)}\right] - d_a = 0 \tag{5}$$

UNITED THERAPEUTICS CORP., EX1034, page 13
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

As $f_b^{-1}f_a(D_a)$ may often be given an explicit algebraic form (in any case, it may be read off the dose-response curves of A and B), equation 5 can be solved to give D_a as will be described below. The expected effect of the combination is then simply $f_a(D_a)$.

A further simplification is possible when all the dose-response curves of the agents in a combination are similar, i.e., they can be described by scaled versions of the same algebraic function and are thus superimposable simply by changing the scale of the dose-axis when this is linear. Suppose the dose-response curve for B is superimposable on that for A by expanding the dose-axis k-fold. Then, for isoeffective doses D_a and D_b for any level of effect, $D_a = kD_b$ and, from equation 4,

$$D_a = d_a + kd_b, \text{ where } k = \frac{D_a}{D_b} \tag{6a}$$

Thus,

$$E(d_a, d_b) = f_a(D_a) = f_a(d_a + kd_b) \tag{6b}$$

Generalisation to combinations of more than two agents is simple (44) and gives

$$E(d_a, d_b, d_c, \cdots) = f_a\left(d_a + \frac{D_a}{D_b} \cdot d_b + \frac{D_a}{D_c} \cdot d_c + \cdots\right) \tag{6c}$$

That is, the effect of a zero-interactive combination of agents with similar dose-response curves is the effect of the sum of the appropriately scaled individual constituents. An analogous equation was derived by Finney (175, 176) for the particular case of combinations of agents with similar probit mortality curves that were linear with log dose.

1. Linear dose-response curves. Effect summation. Biological phenomena are typically nonlinear but, when the observed effect of an agent is only a small fraction of the measurable range, it is generally impossible to distinguish between the dose-response curve and a straight line, whatever the true shape of the former (see section VI 6 iii and fig. 15 below). Thus, curves that are for all practical purposes linear are produced in the low dose/low effect region for many agents, including ionising radiation (318), enzyme inhibitors (445), mutagens and agents causing chromosomal abnormalities (23, 157, 329) and environmental carcinogens, especially where there is a large background effect due to other agents (125, 131, 219, 220, 401). Linear relations may also be found for the effects of some antibiotics on bacterial growth rate constants (194, 195) and it is easy to show that, when an agent producing a simple exponential survival curve acts on cells growing exponentially, growth delay is linearly related to dose (42), as illustrated by the effects of alkylating agents and radiation on tumours (128, 323, 355).With linear dose-response curves,

$$E(d) = \alpha d, \text{ where } \alpha \text{ is a constant.} \tag{7}$$

For any isoeffective doses D_a and D_b

$$\alpha_a D_a = \alpha_b D_b \text{so } \frac{D_a}{D_b} = \frac{\alpha_b}{\alpha_a}$$

Substitution in equation 6b gives

$$E(d_a, d_b) = \alpha_a(d_a + \frac{\alpha_b}{\alpha_a} d_b) = \alpha_a d_a + \alpha_b d_b$$
$$= E(d_a) + E(d_b) \tag{8}$$

That is, when agents have linear dose-response curves, the effect of a zero-interactive combination is the sum of the effects of its constituents.

An example of the correct use of the summation method is the work of Livingston & Dethlefsen (329) on the production of sister chromatid exchanges by X-rays and hyperthermia on cells in vitro (fig. 12). The dose-response curves are sufficiently close to linearity and therefore the effect of a zero-interactive combination should be the sum of the effects of its constituents. A 20 min exposure to hyperthermia induced 5 SCE/cell while 100 rads X-rays induced 3. Thus, a combination of both should have induced 8 SCE/cell. The observed yield was 8.7. Similarly, a combination of 20 min hyperthermia with 200 rads (which induced 6 SCE/cell) should have given 11 SCE/cell. The observed number was 13.1. These results suggest slight synergy. Other examples of usage that may provisionally be regarded as correct (i.e., in the low dose/low effect range where curves may be assumed to approximate sufficiently to linearity) are provided by Reif (418).

Incorrect use of the summation method, including analysis of variance (i.e.,with nonlinear curves) is exemplified by refs. 52, 63, 150, 159, 228, 233, 264, 283, 288, 310, 321, 348, 345, 349, 361, 372, 399, 498, 502, 549, and 550, and there are many other papers in which the summation method is used without information adequate to determine the form of the dose-response curve.

2. Simple exponential dose-response curves. Survivor multiplication. Exponential dose-response curves are typically found in cell survival experiments with ionising and non-ionising radiation and other agents that damage DNA, particularly alkylating agents (4, 37, 38, 100, 128, 145, 154, 161, 162, 180, 227, 295, 311, 377, 434, 453, 454, 492, 498, 532), where the proportions of cells killed and surviving are determined largely by the probability that specific cellular targets will be "hit" by one or more quanta of agent. The result may be consistent with elementary probability theory (taking into account such factors as number of targets per cell and target volume (4, 130, 161, 162, 390, 443, 505), but cell repair mechanisms may reduce effects calculated in this way, especially at low doses, so producing a shoulder on the curve.

When each cell is killed by a single hit on a single crucial target, a simple exponential survival curve, i.e., without a shoulder, is generated.

UNITED THERAPEUTICS CORP., EX1034, page 14
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

Here

$$S(d) = e^{-\beta d} \quad (9)$$

where S is fractional survival and β is a constant giving the slope of the curve when log survival is plotted against dose.

For isoeffective doses D_a and D_b

$$e^{-\beta_a D_a} = e^{-\beta_b D_b}$$

so

$$\text{so } \frac{D_a}{D_b} = \frac{\beta_b}{\beta_a}$$

Substitution in equation 6b gives

$$S(d_a, d_b) = e^{-\beta_a\left(d_a + \frac{\beta_b}{\beta_a} \cdot d_b\right)} = e^{-\beta_a d_a} \cdot e^{-\beta_b d_b}$$
$$= S(d_a) \cdot S(d_b) \quad (10)$$

That is, when agents have simple exponential dose-response curves, fractional survival from treatment with a zero-interactive combination is the product of the fractional survivals from the treatments with its constituents.

The relation of equation 10 to the equations of probability theory is clear. If $P(A)$ and $P(B)$ are the probabilities of independent events A and B, then the probability that either or both occur is

$$P(A \cup B) = P(A) + P(B) - P(A) \cdot P(B) \quad (11)$$

If Q is the probability that an event does not occur, i.e., $Q = 1 - P$, then, from equation 11,

$$1 - Q(A \cup B) = (1 - Q(A)) + (1 - Q(B)) - (1 - Q(A)) \cdot (1 - Q(B))$$

which reduces to

$$Q(A \cup B) = Q(A) \cdot Q(B) \quad (12)$$

If we then take the conceptual leap of substituting fractional effect E of a drug for probability of occurrence of an event, and fractional lack of effect (i.e., fractional survival S) for probability of non-occurrence, we obtain

$$E(d_a, d_b) = E(d_a) + E(d_b) - E(d_a) \cdot E(d_b) \quad (13)$$

and

$$S(d_a, d_b) = S(d_a) \cdot S(d_b) \quad (14)$$

which is identical to equation 10.

The multiplicative rule of equation 14 is one of the most widely misused criteria for zero interaction. This misuse arises out of the longstanding and widespread misconception that lack of pharmacological interaction is equivalent to independent action in the probabilistic sense. Thus it is supposed that the joint effects of non-interacting agents should be described by the statistical law relating to the joint probability of independent events and therefore that, *irrespective of the dose-response relations of the individual agents*, if they do not interact pharmacologically in producing their effects, then fractional survival from a combined treatment (d_a,d_b) should be the product of the fractional survivals from the individual treatments d_a and d_b. Synergy and antagonism are then defined as departures from this condition (151, 215, 235, 243, 357, 397, 411, 513, 531). Now there are indeed many cases in which the effect of a combination of agents satisfies equations 13 or 14 (a good example was provided by Woolfolk and Stadtman (551) for combinations of enzyme inhibitors), and it is correct in such cases to describe the actions of the agents as being independent in the probabilistic sense. However, that does not imply that the effect of the combination is that which is expected from the agents' dose-response curves (zero interaction). This matter will be examined in depth below (section VIII). For the present, it will suffice to note that the supposition that independent action and zero interaction are the same may immediately be invalidated by reference to the preceding section in which it was shown that, when agents have linear dose-response curves, the effect of a zero-interactive combination is the simple sum of the effects of its constituents, and this is not what is indicated by equation 13. It has been shown (44) that equations 13 and 14 apply generally to zero-interactive combinations of a set of agents only when the effects of the individual agents themselves obey the probability laws of equations 11 and 12, i.e., when chance alone determines whether any individual quantum of agent hits its target and a single hit inactivates. Such agents show simple exponential survival curves.

An example of the use of the fractional product method of equation 14 is provided by the work of Bois et al. (60) on inhibition of bioluminescence in *Photobacterium phosphoreum* by combinations of zinc sulphate and penta-chlorophenol, where the curves for fractional residual activity fit equation 9 well (fig. 13a). For example, fractional survival after exposure to 0.6 mg/l Zn was 0.39 and, after exposure to 0.81 mg/l PCP, it was 0.49. The product of these two survivals is 0.19, which agrees well with the observed survival of 0.20 produced by the combination. The isoeffective concentrations of Zn and PCP were 1.04 and 1.88 mg/l respectively, so the interaction index was 0.6/1.04 + 0.81/1.88 = 1.0, and the linear isobole in fig. 13b confirms zero interaction. This is the only example that the reviewer has found in a fairly large literature in which use of the fractional product method was appropriate.

Incorrect use of this method (where the dose-response curves are not simply exponential) may be exemplified by refs. 29, 36, 70, 71, 80, 140, 151, 178, 226, 227, 286, 295, 352, 353, 414, 441, 469, 522, and 538. Some authors commit the additional error of applying the multiplication rule of equation 14 not to fractional survivals but to fractional mortalities (126). This is clearly wrong for, as

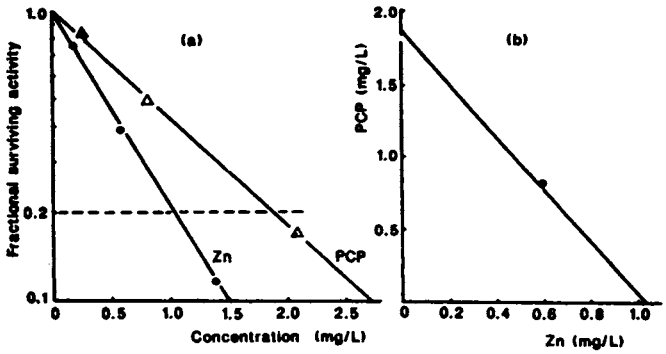

FIG. 13. Use of fractional survivor multiplication as a criterion for interaction (data from table 1 of Bois et al. (60) for effects of zinc and pentacholorophenol (PCP) on bioluminescence in *Photobacterium phosphoreum*). (*a*) Simple exponential dose-response curves for the effects of Zn and PCP on bioluminescence. (*b*) Isobole for the effect of the combination of 0.6 mg/l Zn with 0.8 mg/l PCP. The fractional surviving levels of bioluminescence on exposure to the constituents separately were 0.39 and 0.49, respectively (see (*a*)), and the product of these is 0.19, compared with the fractional level of 0.20 observed for the combination. Concentrations of Zn and PCP isoeffective with the combination were 1.04 and 1.88 mg/l, respectively (read off the curves in (*a*)), so the isobole in (*b*) is virtually a straight line, indicating zero interaction.

each fraction is less than 1, a product of two fractional mortalities will always be less than either one, implying that a combination of drugs is expected to cause less mortality than each constituent alone.

3. Exponential dose-response curves with shoulders. Similar curves. Exponential curves with shoulders may be explained by target theory, taking into account the operation of cellular repair mechanisms and/or multiplicity of cell targets, each of which must be "hit" for the cell to be killed. Using the multi-target expression for cell survival (162), we have

$$S(d) = 1 - [1 - e^{-\beta d}]^n \qquad (15)$$

where β gives the limiting slope of the curve and n is its extrapolation value. Similar curves of this form have equal extrapolation values but may differ in slope and, when $S(D_a) = S(D_b)$,

$$\frac{D_a}{D_b} = \frac{\beta_b}{\beta_a}$$

Substitution in equation 6b gives

$$S(d_a, d_b) = 1 - \left[1 - e^{-\beta_a\left(d_a + \frac{\beta_b}{\beta_a} \cdot d_b\right)}\right]^n$$
$$= 1 - [1 - e^{-\beta_a d_a - \beta_b d_b}]^n \qquad (16)$$

when $n = 1$, (the simple exponential case), this reduces to equation 10.

4. Exponential dose-response curves with shoulders. Dissimilar curves. Here $n_a \neq n_b$, and the curves cannot be superimposed by scaling the dose-axes, so equation 5 is used.

$$f_b^{-1}f_a(D_a) = -\frac{1}{\beta_b}\ln\{1 - [1 - e^{-\beta_a D_a}]^N\},$$

where

$$N = \frac{n_a}{n_b}$$

Thus,

$$D_a\left[1 + \frac{\beta_b d_b}{\ln\{1 - [1 - e^{-\beta_a D_a}]^N\}}\right] - d_a = 0 \qquad (17)$$

The parameters β_a, β_b, n_a, and n_b are obtained from the survival curves and d_a and d_b are given, so D_a is the only unknown in this equation and may easily be found by iteration. The effect of the combination is then found by substituting this value of D_a for d in equation 15 with $\beta = \beta_a$ and $n = n_a$.

5. Linear-log and log-log dose-response curves. In a wide variety of systems, either the response or its logarithm is linear with the logarithm of dose. Agents that may produce such effects include, for example, antimetabolites (37, 469), antibiotics (289), interferons (36, 80, 159, 231, 232, 261), growth factors (372), neuropeptide Y (275), phorbol esters (135), narcotics and neuronal agonists (132, 264, 556), hepatotoxins (404), and cromoglycate (420).The appropriate equations here are:

$$E(d) = \alpha + \beta \log d \qquad (18a)$$

and

$$\log E(d) = \alpha + \beta \log d \qquad (18b)$$

In both cases,

$$f_b^{-1}f_a(D_a) = 10^{(\alpha_a - \alpha_b)/\beta_b} \cdot D_a^{\beta_a/\beta_b}$$

and substitution in equation 5 gives

$$D_a[1 - D_a^{-\beta_a/\beta_b} \cdot 10^{(\alpha_a - \alpha_b)/\beta_b} \cdot d_b] - d_a = 0 \qquad (19)$$

D_a is the only unknown here and may be found by iteration.

6. Sigmoid dose-response curves. i. Mutually exclusive agents. The median effect principle. Sigmoid dose-response curves are generated *inter alia* by agents that obey the law of mass action, for example, drugs that reversibly occupy their receptor sites. Such curves are conveniently analysed using the median effect principle. This was originated by Chou (107) who showed, by examining a variety of examples of first-order inhibition in classical enzyme kinetics that, in general

$$\frac{E(d)}{1 - E(d)} = \frac{d}{M} \qquad (20a)$$

where $E(d)$ is fractional effect of dose or concentration d, and M is the median effective dose or concentration, i.e., that which produces a 50% effect. Equation 20a was subsequently (108) extended to the higher-order case simply by inference from Hill's equation (254, 255) for

UNITED THERAPEUTICS CORP., EX1034, page 16
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

the cooperative binding of oxygen to haemoglobin. This gave

$$\frac{E(d)}{1 - E(d)} = \left[\frac{d}{M}\right]^m \tag{20b}$$

where m is a constant giving the order of sigmoidicity of the dose-response curve and believed to indicate the minimum number of molecular binding sites for the agent. In fact, when there is more than one binding site, observed values for m are often well below the actual number of sites (307).Taking logarithms gives

$$\log (E/(1 - E)) = m(\log d - \log M) \tag{20c}$$

which shows that a graph of $\log (E/(1 - E))$ on the ordinate against $\log d$ on the abscissa is a straight line with slope m and intercept M on the dose-axis. (Alternatively, $\log (S/(1 - S))$ may be plotted, in which case the line has slope $-m$.). This graph is traditionally termed the Hill plot, and has long been used either with $\log(E/(1 - E))$ or equivalent expressions, particularly in enzymology (272, 273, 307, 342), but also in studies of drugs causing muscle contraction (16, 116, 117, 366, 367), neuronal activators (101), inhibitors of cell proliferation (342) and tumour promoters (28), and also in general toxicology (64). The von Krogh equation (519) for agents causing haemolysis is the inverse of equation 20c, and is graphed simply by interchanging the axes of the Hill plot, so that the slope of the line is $1/m$ instead of m. In the literature on the median effect principle, what is usually plotted against $\log d$ is $\log[(1/E - 1)^{-1}]$, but this is simply another way of writing $\log(E/(1 - E)$ and the reasons for preferring the more complicated expression are not clear.

Chou (107, 108) noted that many equations for agents obeying the law of mass action could be put in the form of one or other of the above equations, for instance, the Michaelis-Menten equation for enzyme inhibition, Scatchard's equilibrium-binding equation, the Henderson-Hasselbalch equation for the relation between dissociation and pH and the Langmuir adsorption isotherm, and also pointed out that, in enzyme kinetics, equations based on the median effect principle have the great advantage that they enable fractional effects to be determined without knowledge of kinetic constants or maximum enzyme velocities that are not directly measurable.

From equation 20b, the effect of dose d is given by

$$E(d) = \frac{d^m}{M^m + d^m} \tag{21a}$$

and fractional survival S by

$$S(d) = \frac{M^m}{M^m + d^m} \tag{21b}$$

Chou and Talalay (110, 111) extended this analysis to the effects of combinations of agents, dividing them for this purpose into mutually exclusive and mutually non-exclusive classes. Mutually exclusive agents share the same binding sites and occupation of a site by one agent excludes its occupation by another. As a binding site cannot be occupied simultaneously by different mutually exclusive agents, they cannot interact in producing the effect of interest, and so combinations of such agents should show zero interaction and satisfy equation 1 (fig. 1). Moreover, as the degree of sigmoidicity m ideally reflects the number of binding sites, the dose-response curves of mutually exclusive agents, which share the same binding sites, should have the same value for m (and so should the curves for fixed-ratio combinations of such agents). Thus their dose-response curves are similar, as they can be superimposed by a simple linear scaling of the dose-axis. Further, graphs of $\log(E/(1 - E))$ against log dose for the agents and for their fixed-ratio mixtures will all be parallel.

By considering mass-action mechanisms, equation 22 was derived for this case.

$$\left[\frac{E(d_a, d_b)}{S(d_a, d_b)}\right]^{1/m} = \left[\frac{E(d_a)}{S(d_a)}\right]^{1/m} + \left[\frac{E(d_b)}{S(d_b)}\right]^{1/m} = \frac{d_a}{M_a} + \frac{d_b}{M_b} \tag{22}$$

from which

$$E(d_a, d_b) = \frac{\left(\frac{d_a}{M_a} + \frac{d_b}{M_b}\right)^m}{1 + \left(\frac{d_a}{M_a} + \frac{d_b}{M_b}\right)^m} \tag{23a}$$

and

$$S(d_a, d_b) = \frac{1}{1 + \left(\frac{d_a}{M_a} + \frac{d_b}{M_b}\right)^m} \tag{23b}$$

In fact, when two agents do not interact, equation 22 can be derived directly from the sigmoid function (equation 20b) without invoking mechanisms of action at all, as follows.

For isoeffective D_a and D_b, it is easy to see from equation 20b that

$$\frac{D_a}{D_b} = \frac{M_a}{M_b}$$

Substituting in equation 6a,

$$D_a = d_a + \frac{M_a}{M_b} \cdot d_b \tag{23c}$$

and substituting this in equation 20,

$$\left[\frac{E(d_a, d_b)}{S(d_a, d_b)}\right]^{1/m} = \left[\frac{E(D_a)}{S(D_a)}\right]^{1/m} = \frac{d_a + \frac{M_a}{M_b} \cdot d_b}{M_a} = \frac{d_a}{M_a} + \frac{d_b}{M_b}$$

UNITED THERAPEUTICS CORP., EX1034, page 17
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

which is equation 22.

In the first-order case ($m = 1$), equations 21b and 22 allow the effect of a combination of non-interacting agents to be calculated directly from the effects of its constituents (in a manner analogous to the use of equation 8 for agents with linear dose-response curves and equation 10 for agents with exponential curves), as follows.

From equation 21b, in the first-order case,

$$\frac{1}{S(d)} = 1 + \frac{d}{M}$$

(with appropriate subscripts for agents A and B)

From equation 22, rearrangement gives, in the first-order case,

$$\frac{1}{S(d_a, d_b)} = 1 + \frac{d_a}{M_a} + \frac{d_b}{M_b}$$

Thus,

$$\frac{1}{S(d_a, d_b)} = \frac{1}{S(d_a)} + \frac{1}{S(d_b)} - 1 \qquad (24a)$$

In the case of n inhibitors, the same argument shows that

$$\frac{1}{S(d_1, d_2, \cdots, d_n)} = \sum_{i=1}^{n} \frac{1}{S(d_i)} - (n-1) \quad (i = 1, 2, \cdots, n) \qquad (24b)$$

Equation 24 should be compared with the equation derived by Chou and Talalay (110) relating the initial velocity of an enzyme reaction in the presence of n mutually exclusive inhibitors obeying first-order (Michaelis-Menten) kinetics to the initial velocities in the presence of each of the inhibitors separately. This is

$$\frac{1}{V_{1,2,\cdots,n}} = \sum_{i=1}^{n} \frac{1}{V_i} - (n-1) \qquad (i = 1, 2, \cdots, n)$$

where $V_{1,2,\ldots,n}$ is the multiply inhibited velocity, V_i the velocity in the presence of the ith inhibitor and V_0 the uninhibited velocity. Chou and Talalay (110) showed, by applying the equations of classical enzyme kinetics to a variety of different cases, that this equation held in all, irrespective of the number of inhibitors, whether the reversible inhibition was competitive, non-competitive or uncompetitive, and whether the kinetic mechanism was sequential or ping-pong. However, if the velocities in this equation are converted to fractional velocities S, where $S = v/v_o$, it becomes identical to equation 24. This was derived from equation 22, which in turn was derived without considering mechanisms of action and simply by applying the zero-interaction equation to the sigmoid function. Equation 24 therefore applies, not only to initial velocities of inhibited enzymes, but to the effects of any combinations of non-interacting first-order agents obeying the law of mass action.

It was subsequently shown (112), again by considering mass action mechanisms, that equation 22 could be extended, for mutually exclusive agents, to

$$\frac{d_a}{D_a} + \frac{d_b}{D_b} = \text{combination index} \begin{array}{ll} <1 & \text{(synergy)} \quad (25) \\ =1 & \text{(summation)} \quad (26) \\ >1 & \text{(antagonism)} \quad (27) \end{array}$$

where D_a and D_b are doses isoeffective with the combination at any specified level of effect and "summation" is used in the sense of zero interaction. Equation 26 was termed the General Isobol Equation, and is clearly identical with equation 1 here, and the combination index is identical with the interaction index of equations 1 to 3. Thus, two strictly mutually exclusive agents, behaving according to the law of mass action, do not interact, i.e., the effect of a combination is precisely that expected from the dose-response relations of the individual agents. Considering that equation 26 was derived by examining mechanisms of action while equation 1 was derived by deliberately excluding such mechanisms from consideration, this identity between the two equations is noteworthy, but not surprising. Equation 1 describes the general case of zero interaction and it is to be expected that, when specific cases of zero interaction are analysed in terms of well understood mechanisms, then the equations so derived will be compatible or identical with the general equation. Similarly, Lam (312–315), in analysing the effects of combinations of ionising radiations, the dose-response curves of which do not obey the law of mass action, derived an equation identical to equation 1 to cover this case. These agreements are therefore examples of the general including the particular. It is obviously standing matters on their head to claim (112, 113) that the classical isobole method with equation 1 is a merely a particular case of the median effect principle, that it is the latter which comprises the general case, and that the former may be derived from the latter.

The procedure adopted by these authors to analyse an interaction between mutually exclusive agents is as follows. First, a median-effect plot (that is, a Hill plot) is made of log $(E/(1-E))$ against log dose for each of the agents and for fixed-ratio combinations. These should have correlation coefficients above 0.9. These plots establish the values of m and M to be used in equation 20b and so allow isoeffective doses of the agents and their combinations to be calculated. These values are inserted in equations 25–27 and the combination index is calculated.

Ideally, two agents A and B, mutually exclusive at the same binding site(s), should have dose-response curves of equal sigmoidicities. However, this may not be found in practice, especially when values are determined in

complex in vivo systems, where factors such as differences in metabolism or in access to binding sites may affect the shape of the dose-response curve. This gives rise to a difficulty. When the dose-response curves of two agents have different sigmoidicities, it may not be possible to say, at least without extensive investigation, whether this is because of such complicating in vivo factors or because their binding sites are in fact different (in number as well as location), i.e., because the agents are not mutually exclusive. Equation 32 below was derived to describe the mutually non-exclusive case but Chou and Talalay (112, 113) concluded that, when $m_a \neq m_b$, it was not possible to say whether agents were mutually exclusive or non-exclusive and therefore to decide which of equations 26 or 32 was appropriate Accordingly, they suggested that both equations be used in such cases.

This problem will be discussed further below. The simplest and most direct way to resolve these difficulties is to use the general method described above (figs. 10 and 12), but two algebraic solutions are available, as follows.

From equation 20b,

$$f_b^{-1}f_a(D_a) = M_b\left(\frac{D_a}{M_a}\right)^{m_a/m_b}$$

and substitution in equation 5 gives

$$D_a\left[1 - \left[\frac{M_a}{D_a}\right]^{m_a/m_b} \cdot \frac{d_b}{M_b}\right] - d_a = 0 \qquad (28)$$

As D_a is the only unknown in equation 28, it may be found by iteration, and substitution in equation 20b with the appropriate values of m_a and M_a gives the effect of the combination.

When $m_a = m_b$, equation 28 reduces to equation 23c.

Alternatively, equation 29, suggested by Syracuse and Greco (491), may be used for mutually exclusive inhibitors.

$$\frac{d_a}{\left[M_a\frac{E}{E_{max} - E}\right]}^{1/m_a} + \frac{d_b}{\left[M_b\frac{E}{E_{max} - E}\right]}^{1/m_b} = 1 \qquad (29)$$

The authors did not give a derivation for this equation, but it may easily be obtained from equations 1 and 20b, as follows.

From equation 20b,

$$D = M\left[\frac{E(D)}{1 - E(D)}\right]^{1/m}$$

(with appropriate subscripts for agents A and B)

As $E(D)$ is a fractional effect, it may be replaced by (E/E_{max}), where E is the effect of the combination and $E_{max} = 1$. Thus,

$$D = M\left[\frac{E/E_{max}}{1 + E/E_{max}}\right]^{1/m} = M\left[\frac{E}{E_{max} - E}\right]^{1/m}$$

Using equation 1, equation 29 follows directly, and may be solved by iteration on E.

ii. Mutually non-exclusive agents. Segel (445) showed by kinetic analysis that combinations of mutually non-exclusive enzyme inhibitors that obeyed Michaelis-Menten kinetics were synergistic. Segel did not define synergy explicitly but implied that it meant inhibition greater than would be produced by combinations of mutually exclusive inhibitors at the same specific concentrations. He accounted for this increased effect by the formation of mixed inhibitor-enzyme species that could not be formed by combinations of mutually exclusive agents. Thus, combinations of mutually non-exclusive inhibitors obeying mass action principles have an inhibition mechanism over and above that possessed by mutually exclusive inhibitors. Chou and Talalay (110) also concluded from an examination of enzyme kinetics that the combined effects of mutually non-exclusive inhibitors were necessarily synergistic. An examination of the behaviour of these combinations throws considerable light on two basic issues in this field, namely, the difference between mechanistic and empirical models and the difference between independent drug action and zero interaction.

Consider an enzyme with two binding sites. One site can be occupied by either inhibitor A or inhibitor B, and the other site can be occupied only by inhibitor C. Binding at the site for C is independent of that at the site for A and B, i.e., binding at site C has no effect on binding at the sites for A or B, and vice versa. Thus, A and B are mutually exclusive, whereas C is mutually non-exclusive with respect to A and B. Assume for simplicity that the dose-response curves for all three agents are identical, i.e., that they are described by equation 20b, with the same value for M, and with $m = 1$, that occupancy of either site on the enzyme molecule inactivates it and that, at a concentration M of any one inhibitor, 50% of the appropriate sites are occupied (i.e., M is the median effective concentration). Now examine the consequences, illustrated in fig. 14, of first adding a concentration I of one of these inhibitors, and then a further concentration I of the same or a different inhibitor.

The dose-response curve described by equation 21b, with $m = 1$, shows that, at concentration I of A, B, or C, the fraction of enzyme molecules free of inhibitor (i.e., fractional residual enzyme activity) is $M/(M + I)$. If a further concentration I of the same inhibitor is added, the fraction of molecules free of inhibitor falls to $M/(M + 2I)$. Whether A or B was the first inhibitor added, it makes no difference which of these is the second added, for A and B are completely interchangeable at their joint binding site. Thus, residual fractional enzyme activity with a combination I of A and I of B is again $M/(M + 2I)$. Accordingly, the isobole for fractional residual activ-

UNITED THERAPEUTICS CORP., EX1034, page 19
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

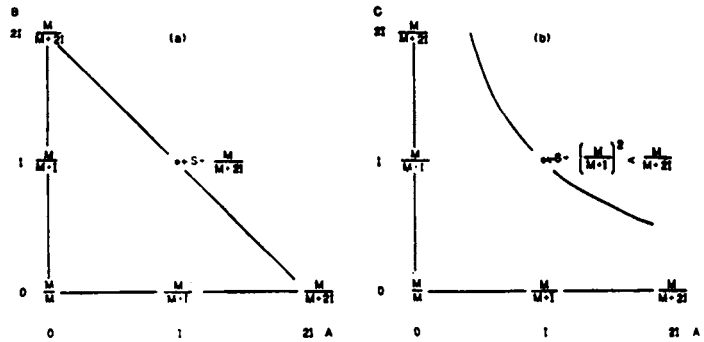

FIG. 14. (*a*) Isoboles for the effects of combinations of *A* and *B* where fractional surviving activity is given by equation 21b with $m = 1$ and the agents are mutually exclusive. The effects of doses $2I$ of *A* and $2I$ of *B* are the same as that of the combination of *I* of *A* with *I* of *B*, and therefore the isobole for this effect is linear. (*b*) Isobole for the effects of combinations of *A* and *C* where fractional surviving activity is given by equation 21b and the agents are mutually non-exclusive. Fractional surviving activity with a dose 21 of either *A* or *C* is again $M/(M + 2I)$ but, from equation 14, fractional survival with the combination of *I* of *A* with I of B is less than this, and therefore the isobole must be concave-up, indicating synergy.

ity $M/(M + 2I)$, which joins the dose $2I$ on the *A* axis with the dose $2I$ on the *B* axis, and which passes through the point representing the combination, will clearly be a straight line, satisfying equation 1 (fig. 14a). Thus, this combination is quantitatively indistinguishable from the necessarily zero-interactive sham combinations (I,I) of *A* or of *B*. Its effect is precisely what is expected from the dose-response curves of the agents and so this combination of mutually exclusive inhibitors is zero-interactive.

However, the result would be different if to the solution containing *A* or *B* were added concentration *I* of *C* (fig. 14c). *C* occupies its own site independently of the situation at the site for *A* and *B*. The fraction of enzyme molecules free of *A* (or *B*) is $M/(M + I)$ and the fraction free of *C* is also $M/(M + I)$ and, as binding at the two sites is independent, equation 14 shows that the fraction free of any inhibitor is $(M/(M + I))^2$. Now, for all nonzero values of *I*, $(M/(M + I))^2$ is less than $M/(M + 2I)$, i.e., fractional inhibition is always greater with the mutually non-exclusive combination of *A* (or *B*) with *C* than with the mutually exclusive combination of *A* with *B*. Accordingly, the isobole for the effect of this combination, i.e., fractional residual activity $(M/(M + I))^2$, does not join the point representing this combination to the points on the dose axes representing 21 of *A* (or *B*) *and* $2I$ of *C*, for these are less inhibitory, but terminates at higher concentrations on the dose-axes, i.e., it is concave-up and does not satisfy equation 1. This combination therefore does not behave like the sham zero-interactive combinations (*I,I*) of *A*, of *B*, or of *C*. Its effect exceeds that expected from the dose-response curves of the agents, and so this combination of mutually non-exclusive inhibitors is synergistic.

This illustrates clearly the difference between expectation based empirically on dose-reponse curves and one based on mechanisms Calculating the *expected* effect of a combination from dose-response curves alone shows how the combination would behave if there were no interaction, and this provides a reference point for evaluating the observed effect of the combination. On the other hand, predicting the *observed* effect requires a sufficient knowledge of the agents' mechanisms of action as well as of the dose-response curves, and such calculations may show that, because of the particular mechanisms of actions of the agents, the combination is synergistic, zero-interactive or antagonistic (215, 268–270, 539, 540). Thus, in the case just considered, examination of mechanisms of action showed that combinations of agents with dose-response curves characterised by equation 20b with m = 1 would be zero-interactive if they were mutually exclusive but that, if they were mutually non-exclusive, they would be synergistic.

This example also shows that independent action and zero interaction are not synonymous, as is often assumed. Combinations of *A* with *B* show zero interaction but not independence, i.e., they satisfy equation 1 but not equation 14. On the other hand, combinations of *A* (or *B*) with *C* show independence but not zero interaction.

For mutually non-exclusive drugs with similar dose-response curves, i.e., $m_a = m_b = m$, Chou and Talalay (112,113) showed that the effect of a combination is given by

$$\left[\frac{E(d_a, d_b)}{S(d_a, d_b)}\right]^{1/m} = \left[\frac{E(d_a)}{S(d_a)}\right]^{1/m} + \left[\frac{E(d_b)}{S(d_b)}\right]^{1/m} + \left[\frac{E(d_a)\cdot E(d_b)}{S(d_a)\cdot S(d_b)}\right]^{1/m} = \frac{d_a}{M_a} + \frac{d_b}{M_b} + \frac{d_a\cdot d_b}{M_a\cdot M_b} \quad (30)$$

As shown by Chou and Talalay (112), when $m = 1$, putting $E = 1 - S$ and rearranging gives $S(d_a, d_b) = S(d_a)\cdot S(d_b)$. That is, in the first-order case, equation 30 is formally equivalent to equation 14 for independently acting agents.

Equation 30 was extended (89, 90) by analogy with equations 25 to 27 to define a combination index for mutually non-exclusive inhibitors as follows.

$$\frac{d_a}{D_a} + \frac{d_b}{D_b} + \frac{d_a \cdot d_b}{D_a \cdot D_b}$$

$$= \text{combination index} \quad \begin{array}{ll} <1 & \text{(synergy)} \quad (31) \\ =1 & \text{(summation)} \quad (32) \\ >1 & \text{(antagonism)} \quad (33) \end{array}$$

However, this extension by analogy is incorrect. The authors have fallen into the trap discussed at the outset (section II), i.e., the idea that a zero-interactive combination is one in which observed behaviour agrees with that calculated from a knowledge of mechanisms of action. However, interaction is defined as departure from expectation based on the dose-response relations of the agents, not expectation based on their mechanism of action. What is expected from dose-response relations is indicated by equation 1 (or the identical equation 26 of Chou and Talalay), and the mechanisms of action of the agents and whether they are mutually exclusive or non-exclusive are irrelevant to this assessment. Thus, the error in the formulations of equations 31 to 33 arises out of the reasoning that equation 30 describes zero interaction between mutually non-exclusive agents because it is derived from an understanding of their mechanisms of action. In fact, equation 30 describes synergy, not zero interaction, and this is just what would be expected from the mechanisms of action of these agents. As shown by Segel (445), by Chou and Talalay themselves (110), and again above (fig. 14b), combinations of first-order mutually non-exclusive agents obeying the law of mass action are necessarily synergistic and, as will be shown below, combinations of higher-order agents are also synergistic at higher dose levels.

Calculating combination indices according to the incorrect equations 31 to 33 has the following consequences. As Chou and Talalay observe, equation 32 has, in comparison with equation 26, an extra term on the left. Thus, this equation always overestimates the combination index and, in consequence, combinations that are in fact synergistic tend to be wrongly categorized as showing zero interaction or even antagonism, and zero-interactive combinations are wrongly deemed to show antagonism.

The idea that different equations for zero interaction are applicable, depending on whether the agents are mutually exclusive or non-exclusive, has also caused difficulties for Chou and Talalay in knowing how to assess combinations of agents that cannot confidently be classified as either, i.e., where $m_a \neq m_b$. It has been suggested (112, 113) that both equations 26 and 32 be used in such cases, on the grounds that the assumption of non-exclusivity (equation 32) would always predict less synergy than that of mutual exclusivity, i.e., that it was conservative in respect of classifying interactions as synergistic. Conservatism is not used here in the usual sense of fixing confidence limits but in the sense of a bias towards classifying interactions as antagonistic and against classifying them as synergistic. Many authors have adopted this unsatisfactory course (98, 99, 113, 167, 189, 232, 415, 464a, 499), and have treated this inherent bias as if it were somehow a virtue, but it has not been explained why under-classification in one direction is an advantage while over-classification in the other is not a disadvantage. In fact, these problems, and the suggested methods for dealing with them, are all quite unnecessary, as they arise out of the misconception described above, i.e., that the definition of zero interaction depends on mechanisms of action. On the contrary, zero interaction is described unequivocally by equation 1, and whether dose-response curves have equal or unequal sigmoidicities is irrelevant in this context. Thus, Chou and Talalay's equations 25 to 27 are applicable in all cases (they are the equivalent of equations 1 to 3 above).

iii. Problems of curve-fitting. The median effect principle has been applied to a wide range of agents, often in circumstances in which simple mass action principles are not known to operate or, indeed, are unlikely to operate. Chou and Talalay (112) therefore sensibly recommended that the method should be used only when there is a high correlation ($r > 0.9$) between log ($E/(1 - E)$) and log dose. However, this recommendation has interesting implications, because one and the same set of data may be closely fitted by a variety of equations. The question naturally suggests itself—if an equation fits, may it be used to calculate the expected effect of a combination, irrespective of the nature of the equation and irrespective of what it may suggest about underlying mechanisms of action? In principle, the answer should be yes, because interaction or the lack of it are determined from quantitative relations between dose and response, not from mechanisms of action, as explained above. A simple concrete example will illustrate this point.

Table 1 and fig. 15 show fractional survival of cells exposed to three different concentrations of a toxic agent. The points are equally well fitted by linear, exponential, and first-order sigmoid curves, with near perfect correlation in all three cases. It follows from the argument above that the calculated effect of a necessarily zero-interactive sham combination of 0.1 mg/ml with 0.2 mg/ml (which must equal the observed effect of 0.3 mg/ml) should be obtained correctly whether we use for the calculation equation 8 (for linear curves), equation 10 (for exponential curves), or equation 24a (for first-order sigmoid curves), and this is indeed the case. In fact, there is only a negligible divergence between observed and calculated effect, whichever of these equations is used. Thus, if the equations fit the data well over the whole range of interest (i.e., over the range of effects caused by the tested concentrations of the single agents *and* the expected effect of the combination), it does not matter which equation is used.

However, this does not imply that equations may be

UNITED THERAPEUTICS CORP., EX1034, page 21
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

TABLE 1

The same set of data may be equally well fitted by linear or exponential survival curves or by Hill plots

Dose (d)	Observed fractional survival (S)	Fractional survival from fitted equations: Linear ($E = 0.827d$, $S = 1-E$ (equation 7))	Exponential ($S = 10^{-0.368d}$ (equation 9, with base 10))	Sigmoid (Hill plot) ($\log [S/(1-S)] = 0.987$ ($\log d$-$\log 1.073$))
0	1.000	1.000	1.000	
0.1	0.909	0.917	0.912	0.909
0.2	0.833	0.835	0.833	0.836
0.3	0.762	0.752	0.760	0.775
Correlation coefficient				
a) Between observed and calculated S		0.998	1.000	0.999
b) Between S and d, $\log S$ and d, and $\log (S/(1-S))$ and $\log d$		−0.998	−1.000	−1.000

Combination	Observed S	Fractional survival calculated for combinations: Equation 8 with $S = 1-E$	Equation 10	Equation 24b
0.1 + 0.2	0.762	0.742	0.757	0.769
0.3 + 0.3 + 0.3	Outside measured range	0.286	0.442	0.516

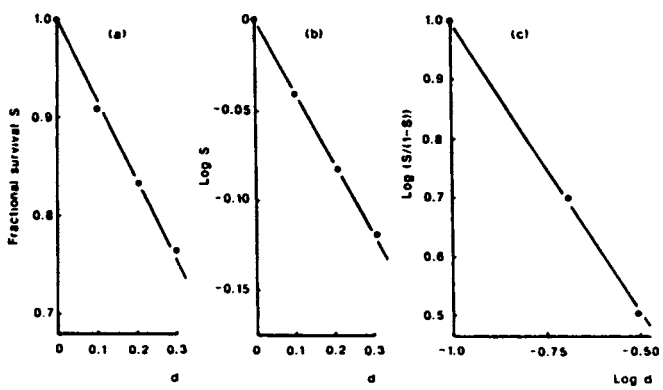

FIG. 15. Three different equations may fit the same data. Fractional survivals with doses 0.1, 0.2, and 0.3 of the agent are given in table 1. (*a*) Survival S plotted against dose d; (*b*) Log S plotted against d; (*c*) Log $(S/(1-S))$ plotted against log d (Hill plot). All curves fit the data well.

used outside the range over which they have been shown to hold. For example, the expected effect of a sham combination of 3 × 0.3 mg/ml will be 0.286 in the linear case, 0.442 in the exponential case, and 0.516 in the sigmoid case. Clearly, at least two of these estimates must be grossly inaccurate.

This underlines the importance, in all procedures that depend on fitting curves to experimental data, of avoiding without good reason extrapolation outside the range of effects over which all the dose-response curves have been shown to fit. Thus, in using the median effect principle, it is not sufficient that the individual curves each fit equation 20c with a high correlation coefficient. It is also necessary that the effect of the combination lies within the common range over which these curves have been shown to fit, because there can be no guarantee that they fit (and thus that median effect equations apply) outside this range. Some authors clearly ignore this elementary requirement, and it is unfortunate that computer-drawn graphs of changes in combination index with fractional effect often cover the entire range of possible effects from zero to complete inhibition, although the range over which dose-response curves are determined is always less (and sometimes much less) than this.

This requirement is particularly important when one or more of the agents used is such that it would not be expected to obey the law of mass action, for instance, it may be an alkylating agent which would be expected to show an exponential survival curve (1a, 64–66, 153–155). Although the data for such agents may fit equation 20c well over the limited range used in the particular experiment, marked divergence is to be expected outside that range. Even worse is the practice of using the median effect principle where the data are clearly insufficient for determining the form of the curve (442, 445, 499). One reason for this cavalier misuse may be the availability of computer software that seems to have seduced some investigators into having these calculations made automatically, without prior thought as to whether they were indeed appropriate to the data.

iv. Application of the mass-action law to complex biological systems. It is worth pointing out that, whereas deductions about mechanisms of action from observed parameters such as the degree of sigmoidicity of a dose-response curve may well be correct when applied in the simple circumstances in which mechanisms are sufficiently well understood (for instance, the effects of reversible inhibitors on enzymes in solution), such deductions are questionable when applied to the much more complex phenomena of cell survival in culture (1, 64–66, 153–155, 258, 304, 415), carcinogenesis (109), viral replication in cells in vitro (231, 232, 518), tumour regression

UNITED THERAPEUTICS CORP., EX1034, page 22
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

in vivo (499), protection of animals against viral infection (442, 471), or killing of populations of insects by insecticides (112, 113). It would be surprising, for example, if the sigmoidicity of the dose-response curve observed in a population of cells or animals bore a direct relation to that of the agent at its molecular site of action and reflected the operation of the law of mass action at that site. As pointed out by Bliss, Finney and others in work to be discussed below, such curves may rather reflect the population distribution of "tolerances," i.e., doses or concentrations of agents required to produce some all-or-none effect, such as death in individual cells or animals. Such population distributions may reveal little about the continuous dose-response curve for the underlying effect, whatever that may be, that leads to death in the individual.

The law of mass action applies strictly to homogeneous and (in principle) unbounded systems of freely diffusible molecules. This is a far cry from the situation within a cell, where the reacting molecules are in large part immobilised on membranes or sequestered in organelles, with the result that the operation of the mass-action law is considerably modified by conformational, partitioning, microenvironmental and diffusion effects (211). Moreover, even if within a cell or organelle reacting molecules were to be regarded as freely diffusible, binding relationships will differ substantially from those predicted by the mass-action law when binding sites are limited (51). These complexities are, of course, raised to orders of magnitude higher where multicellular individuals are concerned, and it difficult to see how it can be seriously suggested that carcinogens, for example, exert their effects on populations of such individuals according to the principle of the mass-action law (109). Even if, in spite of these considerations, such a suggestion were to be entertained, it is a matter of simple observation that, except at the extremes of 0 and 100% effect, any given dose or concentration of agent applied to a population of cells or individuals produces the effect being measured (death, tumour induction, protection from infection, etc) in only a fraction of the population. This shows that the population is heterogeneous in its response and suggests strongly that heterogeneity, rather than any simple chemical law, may determine the form of the dose-response curve.

An instructive example is afforded by the dose-response curves for lysis of red cells by various agents (bacterial lysins, complement, alkali, hypo-osmolar solutions, etc). These curves are sigmoid and are transformed to straight lines when log(E/(1 − E)) is plotted against log concentration of lytic agent (519), and thus might without more ado be accepted as manifestations of the mass-action law. In fact, however, the red cell population is heterogeneous in susceptibility to lysis because it is heterogeneous in age, and susceptibility is age-dependent. Indeed, differential osmotic lysis may be used to separate red cell subpopulations of different ages (351, 459). Thus, these dose-response curves are manifestations of population heterogeneity, not of the law of mass action. The dose-response curves for killing of insects by insecticides, often used to illustrate the median effect principle (112, 113, 201) are likely also to reflect population heterogeneity, as are other effects measured on populations (98–100, 109, 114, 153–155, 231, 232, 258, 304, 415, 442, 471, 499, 518).

Figure 16 illustrates how a linear Hill plot may be generated simply by a population distribution. It shows that, when susceptibility in a population to some all-or-none effect such as death is log-normally distributed with respect to dose, log $(E/(1 - E))$ is highly correlated ($r = 0.998$) with log dose over at least a 5-log range of dose and over a fractional effect range from 0.006 to 0.99. A standardised normal distribution was used to generate the curves in this figure, and gave Hill plot values of $M = 1.0$ and $m = 0.83$. Other values for these parameters would be obtained by using distributions with different means and standard errors.

These examples do not mean that equations based on the median effect principle may not be used for quantitation in complex systems, if they fit the data. However, they provide a salutary warning against using a mere fit (however good) to an equation to make detailed inferences about supposed mechanisms of action, whether these are based on the mass action law, whether agents are mutually exclusive or non-exclusive, and so on, when in reality all such suppositions may be illusory and the

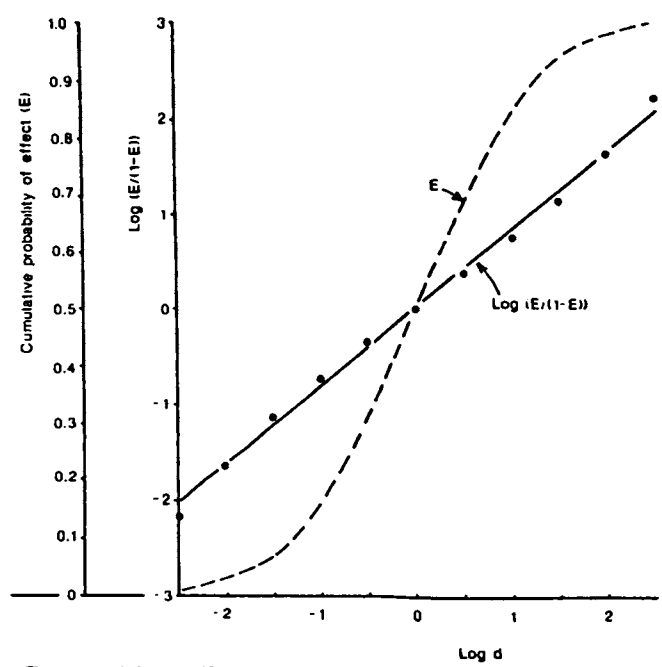

FIG. 16. A linear Hill plot may be generated when susceptibility to a quantal effect (e.g., death) is log-normally distributed with dose. A standardised normal distribution on a logarithmic dose scale was used to generate the curve of cumulative probability of effect, which is sigmoid with log dose. A Hill plot of log$(E/(1\text{-}E))$ is linear with log dose over a 5-log range ($r > 0.99$).

fit may reflect only a distribution of sensitivities between individuals.

v. Utility of the median effect principle. In summary, the importance of the median effect principle is that it enables calculation of the effects of combinations of agents that obey the law of mass action. It is particularly useful in the study of enzyme inhibitors, for it enables effects to be calculated without reference to kinetic constants. The method, including the Hill plot, may also be used whether or not the agents are known to obey the mass action law, provided the dose-response curves fit equation 20c well over the range of interest, and provided that the effect of the combination lies within the range over which all the single agent dose-response curves have been shown to fit the equation. (It should not be assumed, however, that a fit to this equation implies that the agents obey the law of mass action).

An integral step in the procedure advocated by Chou and Talalay is the calculation of correlation coefficients that determine the applicability of the equations to the data. This step could with advantage be adopted more widely with respect to curve-fitting when other types of equations (linear, exponential, etc.) are applicable.

The procedure requires that a series of combinations be tested in which the agents are present in fixed ratio. This requirement arises because (*a*) the doses of agents in combinations producing specified effects are determined from equation 20b, so that it is necessary to determine m and M for combinations as well as for agents, and (*b*) the authors believe that the choice of equations to be used for assessing the interaction depends on whether the agents are mutually exclusive or non-exclusive. However necessary this requirement may be for the procedure advocated by Chou and Talalay, it is not necessary for the assessment of interactions. As has been shown repeatedly above, an interaction index may be calculated for an individual combination, irrespective of whether others are also tested and of their arrangement, and mutual exclusivity or non-exclusivity is irrelevant to the calculation.

When each of the dose-response curves fits equation 20c but sigmoidicities are unequal, equations 28 or 29 may be used to calculate the effect of a zero-interactive combination, but the mechanism-free general method described earlier (figs. 10 and 12) is preferable as it avoids the requirement to fit equations to data that may not necessarily be well-behaved.

The criteria used by these authors to evaluate interactions between mutually exclusive agents (equations 25 to 27) are correct, for they are in fact those of the classical isobole method (equations 1 to 3) of which they represent a particular case. As shown above, these equations are valid irrespective of mechanisms of action or of the forms of dose-response curves. Thus, these criteria are valid for agents of all types, and are not, as these authors suppose, restricted to mutually exclusive agents. For the same reasons, computer-drawn plots of combination index versus fractional effect are correct when based on the assumption of mutual exclusivity (equations 25 to 27), whether the agents are mutually exclusive or not, for these equations assign an index of 1 to zero-interactive combinations. Such plots should not be extrapolated outside the range of effects common to all the single agent dose-response curves.The criteria advocated for evaluating interactions between mutually non-exclusive agents (equations 31 to 33) are incorrect, for they are based on equation 30, which describes the behaviour of mutually non-exclusive agents that would be expected from their mechanisms of action, not the behaviour to be expected if they did not interact, which is described by equation 1 (or equation 26). For the same reasons also, plots based on the "conservative" assumption of mutual non-exclusivity (equations 31 to 33) are incorrect, for they wrongly attribute zero interaction to inherently synergistic combinations and thus overestimate the degree of antagonism.

VII. Additivity Envelopes

The more or less intuitive origins of the isobole method and the uncertainty of many leading investigators as to whether it could be applied to combinations of agents with dissimilar dose-response curves have understandably led to attempts to "improve" the method. One such attempt that has had a certain vogue is the additivity envelope method of Steel and Peckham (479, 480). These authors wrongly assumed that linear isoboles indicated zero interaction only when the individual agents in the combination had linear dose-response curves (or curves that could be linearized by an appropriate transformation). Of course, any continuous curve that is not closed can be linearized by a suitable transformation, just as any piece of string can be straightened, so these authors added the proviso that the transformation should be to a quantity with some "biological meaning." They did not, however, say how mathematical transformations with this property could be distinguished from those without it.

The field in which these authors were primarily interested was radiobiology, in which the dose-response curves of main interest are exponential survival curves, usually with shoulders. According to Steel and Peckham, the shape of the zero interaction isobole was uncertain in such cases, and they proposed to set bounds to this supposed uncertainty by using the extremes of the survival curves of the agents, i.e., the initial shoulders (Mode I calculation) and the limiting slopes (Mode II calculation) to construct an envelope that would assuredly contain the zero interaction isobole. Points below and to the left of the envelope would therefore represent synergistic combinations and points above and to the right antagonistic combinations.

In fact, as shown for two-agent combinations above, and for the general case elsewhere (44), the shape of the

dose-response curve is irrelevant to the construction of the zero interaction isobole and no uncertainty arises on that score. However, the additivity envelope is certainly valid under specific conditions, and it is instructive to see what these are.

The Mode I calculation may be made as follows. Suppose a combination (d_a,d_b) produces fractional cell kill $K(d_a,d_b)$. On a graph of the survival curves, the effect of dose d_a of agent A is measured by the vertical distance representing cell kill $k_a(d_a)$ due to that dose alone. Then a point is located on the survival curve of B such that $k_a(d_a) + k_b(d_b) = K(d_a,d_b)$. In other words, if effects are defined in terms of these distances only (on whatever scale is appropriate), it is assumed that the "effect" of a zero-interactive combination is the sum of the "effects" of its constituents. But this is true in general only when, for both agents, "effect" is linearly related to dose (44). Thus, if cell kill is measured on a linear scale, kill is simply proportional to dose and, if it is measured on a logarithmic scale, effect is a simple exponential function of dose. In either case, the survival curves would be linear on the appropriate scales. Thus, the Mode I calculation is based on the assumption that the survival curves of both agents can be linearised by appropriate choice of effect scale. This point is worth emphasising for, when an agent has a nonlinear dose-response curve, even sham combinations of the agent with itself generate curved Mode I isoboles (42).

The Mode II calculation is made by following the two survival curves sequentially as follows. (1) The effect of d_a is first found from the curve for A. (2) The point corresponding to this effect is located on the curve for B. (3) An increment equal to d_b is measured from that point along the curve for B to give the expected effect of the combination. (4) This last manoevre is equivalent to displacing the corresponding point on the curve for B to the left by a distance d_b.

In mathematical terms, this sequence of steps may be analysed by treating effects as monotonic (invertible) functions of doses and supposing that D_a and D_b are respectively doses of the individual agents that are isoeffective with the combination. (1) The first step entails finding $f_a(d_a)$. (2) In the second step, the dose of B isoeffective with d_a is $f_b^{-1}f_a(d_a)$. (3) Adding an increment d_b of B to this so that the total amount of B is isoeffective with the combination (and therefore with D_b) implies that

$$f_b^{-1}f_a(d_a) + d_b = D_b$$

So, using equation 1,

$$f_b^{-1}f_a(d_a) = D_b - d_b = \frac{D_b}{D_a}\, d_a$$

Inverting the monotonic function f_b^{-1} gives

$$f_a(d_a) = f_b(kd_a), \quad \text{where} \quad k = \frac{D_b}{D_a}$$

From elementary algebra, the curve for the function $f(kd)$ is simply the curve for $f(d)$ with the d-axis expanded k-fold. Accordingly, the method of following survival curves sequentially to find the expected effect of a zero-interactive combination is valid only when the curves are superimposable by a linear expansion or contraction of the dose-axis, i.e., when they are similar.

When the conditions for the validity of the Mode I and Mode II calculations are both satisfied (the former is only a special case of the latter), both calculations produce the same linear isobole, i.e., the envelope collapses to a single straight line, and this is precisely the isobole produced by the classical isobole method. Illuminating examples, showing almost complete collapse of the envelope to a single straight line for combinations of agents with almost linear dose-response curves (on a log-linear plot) are given by Siemann *et al.* (453, 454). The originators of this method pointed out that the advisability of using it depended on the closeness of the survival curves to linearity on the appropriate scale (479, 480). This advice is almost always honoured more in the breach than in the observance, but it is fully supported by the above analysis. It appears, therefore, that the envelopes that several authors have depicted (137, 190, 246, 281, 295, 370, 387, 415, 485, 486, 492, 494, 497, 498, 504) are no more than artefacts occasioned by application of the method to non-linearized curves (artefacts that may be produced even for sham combinations of one and the same agent). When used in this fashion, the artefacts may include, for example, a concave-down lower bound to the envelope (137, 281, 295, 370, 492, 494), which could result in antagonistic combinations being deemed synergistic, and a concave-up upper bound (485, 504), which could lead to synergistic combinations being deemed antagonistic. The experiments of Tsai et al. (504) show how misleading these artefacts may be. These authors were interested in the observation that combinations of etoposide and cisplatin were effective in variety of human lung tumours and had shown therapeutic synergy in a mouse tumour model. They therefore set out to see whether these favourable features could be attributed to synergy that might be demonstrable in vitro. Eight human lung tumour cell lines were tested, and a total of 32 isobolograms was generated. Most (29/32) of these showed moderate to marked synergy according to the classical isobole method. However, the lower bounds of the additivity envelopes were all concave-up, sometimes markedly so, with the result that the great majority of combinations fell within the envelopes. Thus, although synergy between the two drugs was plain to see on simple inspection of the isobolograms, the idea that the additivity envelope method consitituted a "rigorous test" for synergy compelled the authors to ignore this clear evidence and to conclude that there was no overall synergy, and therefore that the clinical benefit of combinations of these drugs was not due to synergy at the cell level.

UNITED THERAPEUTICS CORP., EX1034, page 25
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

If the additivity envelope method is applied to linear or linearized curves only, these artefacts do not appear, and conventional straight line isoboles are produced, identical to those of the much simpler classical isobole method. The envelope additivity method therefore serves no useful purpose and should be discarded.

VIII. Similar and Dissimilar Action. Independence and Zero-Interaction.

Concentration-and Response-Addition

We now return to a more detailed discussion of a problem raised earlier. An important fallacy discussed in section II was that interactions between agents should be defined in terms of their mechanisms of action. A corollary of this is that interactions occurring between agents with similar mechanisms of action should differ in some basic way from those between agents with dissimilar mechanisms, that this difference should be apparent when the interactions are analysed quantitatively, and therefore that different methods should be used for analysing interactions between agents with similar actions on the one hand and between those with dissimilar actions on the other.

The idea was first clearly expressed by Bliss (56), Finney (175, 176), and Plackett and Hewlett (405), who described two types of non-interaction: (*a*) similar action,in which drugs were supposed to have the same mechanisms of action, to act at the same sites and to behave in all respects as simple dilutions of each other and (*b*) independent action, in which drugs were supposed to act at different sites with different mechanisms of action.

When drugs act as simple dilutions of each other, their dose-response curves should be similar, i.e., when doses are on a linear scale, the curves should be superimposable by a simple linear scaling of the dose axis and, when the dose axis is logarithmic, the dose-response curves should be parallel. It is easy to determine the nature of the isoboles that would be generated by non-interacting combinations in such cases. If drug B behaves as a k-fold dilution of drug A then, in equation 1 we simply substitute $k^{-1}d_b$ for d_b and $k^{-1}D_b$ for D_b and the equation is thereby unchanged. Accordingly, such combinations generate linear isoboles. Moreover, as the two dose-response curves are superimposable, k is constant over the whole range, so the isoboles for different levels of effect are parallel (fig. 1).

On the other hand, when drugs act dissimilarly, then it was supposed that this would usually be reflected in a dissimilarity of their dose-response curves, i.e., these would not be superimposable by any linear scaling of the dose-axes and linear-log plots would not be parallel. It was postulated that, when such drugs do not interact, then they may be said to act independently, and therefore that the effects of combinations may be derived directly from statistical theory for independent events. This reasoning implies that the effect of a zero-interactive combination of A and B will be given by equation 13 (that is, by equation 11 with fractional effects substituted for probabilities). Alternatively, if lack of effect is being measured (i.e., fractional survival of enzyme activity, of cell populations, etc), then equation 14 would hold (which is equation 12 with fractional survivals substituted for probabilities). Departures from these two types of non-interaction were supposed to indicate synergy or antagonism ("negative synergy").

It soon became clear that there were difficulties with this approach, and it was subsequently substantially elaborated and modified (18–22, 177, 247, 248, 249, 250–252, 406–408). However, these modifications, which were largely concerned with constructing mathematical models to fit various types of interaction and non-interaction, are not directly relevant here, as it is the original unmodified formulation that has had such a notable influence in this field. Some of the methods described above show this influence, and it is useful to reconsider these together.

(*a*) Loewe (225) divided combinations into those in which the dose-response curves were "homodynamic" (i.e., similar) and "heterodynamic" (i.e., dissimilar). This classification was based on an earlier paper by Frei (185) and was apparently made independently of the work of Bliss and Finney, but it is clearly analogous. Loewe concluded that isoboles for non-interactive combinations would be linear in the former case but that, in the latter, they would generally be nonlinear. However, the reasoning behind the latter conclusion was obscure. Loewe found further that the equation he proposed for non-interacting heterodynamic combinations would generate two different isoboles for the same effect, although both common sense and common observation show that, for combinations of agents with monotonic dose-response curves, each effect can have only a single isobole. Possibly the intention was to imply that the position of the isobole was uncertain in such cases (not that there would in reality be two isoboles), but the paper is not clear on this point. In view of these difficulties, it is hardly surprising that Loewe concluded that synergism and antagonism were "imaginary magnitudes devoid of a basis of reference and of practical applicability." This conclusion happily seems not to have deterred him or most other investigators from using the isobole method, which rests firmly on the idea that synergy and antagonism are most certainly real, have an unequivocal basis of reference and are indisputably of practical use.

(*b*) Traditional radiobiological criteria for interactions divide them into four main classes (145, 227, 443, 508) defined by the effect of a fixed dose of one agent on the dose-response curve (generally the curve of log survival) of the other. These classes are (i) independence, in which a fixed dose of agent B shifts the log survival curve of A uniformly downwards by a fixed distance, and the frac-

UNITED THERAPEUTICS CORP., EX1034, page 26
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

tional product equation 14 holds; (ii) additivity, in which a fixed dose of B shifts the survival curve of A uniformly to the left on a linear scale (this criterion for additivism was also used by Draskoczy (149) and Pöch (409–412); (iii) synergy, in which a fixed dose of B increases the slope of the survival curve of A, i.e., the displacement downwards increases with the dose of A; and (iv) antagonism, in which the slope of the survival curve is decreased.

"Independence" as defined by these criteria clearly corresponds to the independent case of Bliss. In the so-called "additive" case, it is supposed that "the damage due to a dose of B has the same effect on the survival curve due to A as a survival equivalent dose of A" (227) or "B is looked at as if it were an unknown dose of A" (412). This case is thus equivalent to the similar action case of Bliss, and it is clear that both "independence" and "additivity" are meant to be types of non-interaction, distinct from the interactions represented by the remaining two classes. However, the originators of this classification seem not to have appreciated that simple downward or horizontal shifts in the survival curve would be produced by zero interaction only when these curves had certain specific properties.

As shown above, the independent case, described by equations 10 and 14, applies generally to non-interactive combinations of a set of agents only when the agents have simple exponential survival curves. The "additive" case, in which the curve is shifted horizontally on a linear scale, describes zero interaction only when the dose-response curves of the agents are similar. For, if a fixed dose d_b of B shifts the dose-response curve for A to the left by a fixed distance, then this is equivalent to converting the function $f_a(d_a)$ to the function $f'_a(d_a) = f_a(d_a + kd_b)$ where k is a constant. As the effect of the combination equals that of D_a, we have

$$f_a(d_a + kd_b) = f_a(D_a)$$

and therefore, for monotonic functions,

$$d_a + kd_b = D_a$$

If, as supposed, we are here dealing with a case of zero interaction, we may use equation 1 to show that

$$k = \frac{D_a}{D_b}$$

i.e., if k is constant, so also is the ratio between D_a and D_b. The shift in the curve for A is fixed at all effect levels, so this ratio is also fixed at all levels, i.e., the dose-response curves of A and B are similar.Simple exponential survival curves are, of course, similar in the sense used here so, with combinations of such agents, the downward and laterally shifted curves would coincide. This invalidates the idea that the difference between combinations of agents with similar and dissimilar (independent) mechanisms of action is revealed by the different types of shift produced in the curve.

(*c*) Steel and Peckham (479, 480) justified their additivity envelope method by the supposed difference in the pattern of zero interaction shown by agents with linear and nonlinear dose-response curves respectively. However, analysis of the methods by which they calculated the bounds of the envelope shows that the underlying idea is that of differences between agents with similar and dissimilar modes of action and dose-response curves (485,486). Their Mode I calculation entails adding log effects, i.e., it is equivalent to the independent case (dissimilar action in the scheme of Bliss) when agents have simple exponential dose-response curves, and their Mode II calculation requires the dose-response curves to be similar. The fallacies in their procedure have been discussed above.

(*d*) Chou and Talalay (110–113) classified agents that obeyed the law of mass action as either mutually exclusive or mutually non-exclusive, acting respectively at the same or different site(s). The former would ideally have similar dose-response curves (corresponding to the similar case of Bliss) and application of the mass action law showed that non-interactive combinations of such agents would generate linear isoboles. Mutually non-exclusive agents may have dissimilar dose-response curves and Chou and Talalay reasoned that, as they acted at different sites, their actions would be independent (corresponding to the independent case of Bliss), and their equation describing zero-interactive combinations of such agents (equation 30) is, in the first-order case, equivalent to equation 14 for independent action. In fact, there are well-attested examples of independent action of agents that obey the mass-action law, for instance, inhibitors that bind to different sites on the enzyme molecule (551). However, difficulties with the analysis arose because, when agents have sigmoid dose-response curves, equation 30 does not produce linear isoboles. For example, in the first-order case, all isoboles are concave-up because, as discussed above, such combinations are synergistic by virtue of the mechanisms of action of the agents (fig. 14). Unfortunately, Chou and Talalay then equated zero interaction with effects expected from analysis of mechanisms of action (the basic error discussed in section II). This reasoning led them to an incorrect equation for zero interaction (equation 32) for mutually non-exclusive agents, an equation that in fact described synergy, and to the incorrect conclusions (112, 113) that equation 30 "does not describe an isobologram," that combinations of mutually non-exclusive agents that they had previously shown were necessarily synergistic (110) in fact showed summation, i.e., zero interaction, and that the isobole method was applicable only to combinations of mutually exclusive agents.

(*e*) Finney (176) pointed out that similar and independent action could be distinguished on the basis that,

in the former case, the effect of a combination on a population of individuals could be obtained by calculating the effect of the sum of the doses of the constituents (weighted according to potency) whereas, in the latter case, it could be obtained by adding the effects of the constituents (with due subtraction of effects occurring jointly in the same individuals, as in equation 13). Gessner (200, 201) accordingly termed the two cases dose-addition and effect-addition (or effect-summation), respectively. Analogous terms (concentration-addition and response-addition) were coined by Anderson and Weber (8) in order to avoid the connotation implied in Bliss' classification that the modes of action of the agents were known to be similar or independent. (Chen et al. (106) used the term response-addition in a different sense to mean addition without subtraction of joint effects, i.e., as in equation 8.)

Gessner (200, 201) noted that, whereas the dose-addition isobole was always linear, the effect-addition isobole calculated from equation 13 would be convex-down in his experiments (which concerned loss of righting reflex in mice given combinations of d-tubocurarine and ethanol). Weber et al. (533) also found convex-down response-addition isoboles when assaying mortality in fish caused by combinations of heavy metals and pesticides. This type of curvature was noted also by Pöch and Reiffenstein (413). According to the classical isobole method, the isoboles depicted by these authors indicate antagonism. Nevertheless, the conviction that independence and zero interaction were equivalent led them to conclude that such curved isoboles represented a type of zero interaction. This fallacy originates in a failure to realise that the response-addition isobole is not a general indicator of zero interaction, that the shape of this isobole depends on the shapes of the agents' dose-response curves and the doses employed, and that it indicates zero interaction only when the dose-response curves of the agents themselves are described by the independent action equation 13.

That this is so is shown by a more general examination of the differences between mutually exclusive and mutually non-exclusive inhibitors discussed above. Consider a combination (d_a,d_b) where agents A and B have sigmoid dose-response curve described by equation 20b, with equal sigmoidicities m. If the combination is zero interactive (concentration addition), the reciprocal of fraction residual activity is, from equation 23b, given by

$$\frac{1}{S(d_a, d_b)} = 1 + \left[\frac{d_a}{M_a} + \frac{d_b}{M_b}\right]^m \tag{34}$$

On the other hand, if the combination shows independent action of A and B (response addition), the reciprocal fractional activity is, from equations 14 and 21b, given by

$$\begin{aligned}\frac{1}{S(d_a, d_b)} &= \frac{1}{S(d_a)} \cdot \frac{1}{S(d_b)} \\ &= \left[1 + \left(\frac{d_a}{M_a}\right)^m\right] \cdot \left[1 + \left(\frac{d_b}{M_b}\right)^m\right] \\ &= 1 + \left(\frac{d_a}{M_a}\right)^m + \left(\frac{d_b}{M_b}\right)^m \\ &\quad + \left(\frac{d_a \cdot d_b}{M_a \cdot M_b}\right)^m \end{aligned} \tag{35}$$

When $m = 1$, the right-hand side of equation 35 is always greater than that of equation 34, i.e. fractional residual activity is always less with independent action that with zero interaction. Thus, if two first-order inhibitors with identical dose-response curves behaved as mutually non-exclusive inhibitors (independent action) and so generated response-addition isoboles, all combinations would be more inhibitory than expected from zero interaction, i.e., they would be synergistic. Indeeed, this is what was found in such cases by Segel (445) and by Chou and Talalay (110) (and see figs. 14 and 17).

The situation for higher-order cases is more complex and may produce synergy, zero interaction, or antagonism, depending on the relation between d, m, and M (fig. 18). For example, if $m = 2$, the right-hand side of equations 34 and 35 are equal only when $d_a.d_b = 2M_aM_b$ When $d_a,d_b < 2M_a.M_b$, combinations of two second-order inhibitors with equal sigmoidicities would leave less residual enzyme activity in the zero-interactive, concentration addition case (equation 34) than in the independent, response-addition case (equation 35) and, at doses above this level, they would leave more residual activity. Thus, if two such agents behaved as ideal mutually non-exclusive inhibitors and generated response-addition isoboles,

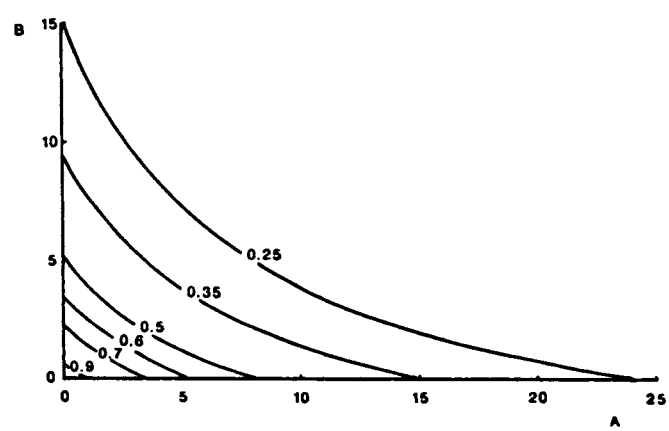

FIG. 17. Isoboles for fractional surviving effects from 0.25 to 0.9 for combinations of first-order mutually non-exclusive inhibitors A and B. The dose-response curve of each agents is given by equation 21b, with $m_a = m_b = 1$, $M_a = 8$, $M_b = 5$. As A and B are mutually non-exclusive, their effects are independent, and therefore the effects of combinations are given by equation 14. All isoboles are concave-up, indicating synergy.

UNITED THERAPEUTICS CORP., EX1034, page 28
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

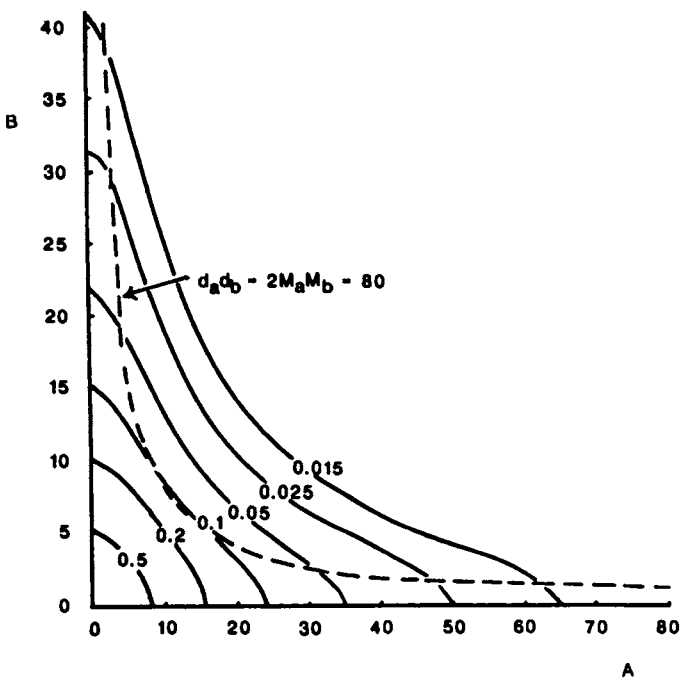

FIG. 18. Isoboles for fractional surviving effects from 0.015 to 0.5 for combinations of two higher-order mutually non-exclusive inhibitors *A* and *B*. The dose-reponse curves are given by equation 21b with $m_a = m_b = 2$, $M_a = 8$, $M_b = 5$, and the effects of combinations by equation 14. Combinations (d_a,d_b) show zero interaction when $d_a d_b = {}^2M_a M_b = 80$, (indicated by the dashed line), antagonism when $d_a d_b$ is less than this and synergy when it is greater.

combinations would show zero interaction when $d_a d_b = 2M_a M_b$, antagonism at lower doses and synergy at higher doses.

The dependence of the response-addition isobole on the shapes of the dose-response curves was shown also by Christensen and Chen (115) and Unkelbach and Pöch (509) for agents with Weibull dose-response curves, where fractional survival is given by

$$S(d) = e^{-\beta d^n} \tag{36}$$

Here, the effect of a combination (d_a,d_b) of agents with identical dose-response curves is, in the zero-interactive (concentration addition) case,

$$S(d_a, d_b) = e^{-\beta(d_a+d_b)^n} \tag{37}$$

and, in the independent (response-addition) case,

$$S(d_a, d_b) = e^{-\beta(d_a{}^n+d_b{}^n)} \tag{38}$$

When $n > 1$, survival in the independent case always exceeds that in the zero interaction case and so, if two such agents behaved with strict mutual exclusivity, generating response-addition isoboles, combinations would show antagonism. Conversely, when $n < 1$, the response isoboles generated by mutually non-exclusive agents with such survival curves would show synergy. When $n = 1$ (simple exponential survival curves), equations 37 and 38 are identical and zero interaction and independence coincide. Thus, when combinations of agents with simple exponential survival curves show zero interaction, the isoboles are linear whether calculated according to "concentration addition" or "response addition."

In each of these five examples, the idea that the pattern shown by zero interaction depends on the mechanisms of action of the agents (specifically, on whether these mechanisms are similar or dissimilar) has led to incorrect conclusions. In fact, if interaction is defined in terms of observed effects (not mechanisms of action, real or supposed), then one pattern describes all zero-interactive cases, and this is shown by the classical isobole method and equation 1. When combinations are non-interactive, the only difference between the isoboles for combinations of agents with similar and with dissimilar dose-response curves is that, in the former case, the linear isoboles for different levels of effect are parallel (fig. 1) whereas, in the latter case, they are not.

The following additional points are relevant in considering these problems.

(*a*) Imprecise usage of the term "independence" has led many to conclude that, if agents act independently (in the probabilistic sense), they cannot interact pharmacologically in producing their effects. This conclusion is incorrect. Independence is *defined* by equation 13 (56, 175), and the effects of combinations that satisfy this equation behave as do the probabilities of events that are statistically independent. Zero interaction, on the other hand, is *defined* by equation 1, and combinations satisfying this equation behave as do sham combinations of one and the same agent, the effects of which are necessarily those expected from the dose-effect relations. A combination may satisfy one or both or neither of these equations. When agents have simple exponential dose-response curves, sham combinations of one and the same agent, which necessarily satisfy equation 1, also satisfy equation 13. Thus, such combinations show both independent action and zero interaction (see also Unkelbach and Pöch (509) and Kodell & Pounds (303)). By the same token, zero-interactive combinations of different agents of this class also satisfy both equations, showing both independent action and zero interaction. On the other hand, a combination may, for instance, satisfy equation 13 but not equation 1, i.e., the constituents act independently in the statistical sense but show pharmacological interaction. This is ideally the case with the combinations of mutually non-exclusive inhibitors discussed above which, as shown by figs. 14, 17, and 18 and by equations 37 and 38, show synergy, zero interaction, or antagonism, depending on the circumstances (degrees of sigmoidicity or slopes of the dose-reponse curves and doses of the agents). A sham combination of various doses of one and the same agent of this class would satisfy equation 1, as it is necessarily zero interactive, but would not in general satisfy equation 13 as the dose-response curve is not exponential.

(*b*) Although a principal motivation of these authors was to introduce mathematical rigour into the study of

drug interactions, their formulations in regard to supposed biological mechanisms were essentially imprecise and equivocal. Non-interaction between agents was said to exist when neither modified the physiological action of the other that led to the response in question (56) and this definition was accepted by Hewlett and Plackett (249), Ashford and Smith (21), and Shelton and Weber (451). However, consider the effect of a sham combination of two doses of one and the same agent, in which individual molecules of agent compete for binding to the same site. Unquestionably, the action of a second dose is modified (i.e., its fractional effect is reduced) by the presence at the site of molecules of drug from the first dose, yet the two doses do not interact pharmacologically as the effect of such a combination is precisely that expected from the agent's dose-effect relations. Or consider the effect on cell survival of two successive doses of an agent which has a shouldered exponential survival curve. Again, the action of the second dose is certainly modified (i.e., its fractional effect is increased) as a consequence of administration of the first dose although, again, the effect of this sham combination can only be that expected from the agent's dose-response curve and thus it shows zero interaction.

It is also appropriate to point out, with reference to supposed mechanisms, that agents that act truly independently of each other do not necessarily act at different sites and with different mechanisms of action. With a single target/single hit inactivation mechanism, such as may be shown by ionising radiations for example, all the quanta of agent act independently of each other in the probabilistic sense, but they all have the same mechanism of action and act at the same site (162, 318).

(*c*) The work of Bliss, Finney, Hewlett, and Plackett was concerned primarily with quantal (all-or-none) effects on populations of organisms and not with continuously variable effects on individual organisms, although attempts have been made to link the two types of response (250, 252) in order to generalize the equations that were derived. However, because of the nature of the experimental material from which these authors initially drew most of their data (killing of insects by insecticides, where probit mortality is linear with log dose), the mathematical treatment was oriented almost entirely to dose-response curves of this particular form, and the extent to which the equations generated can be applied to pharmacological responses in general, in which most dose-response curves are not of this form, is questionable.

(*d*) The dose-response curve of a drug is likely to reflect its primary action closely only when the effect being measured is an immediate or close consequence of the primary action itself. When the observed effect is more or less distanced from the primary action by intermediate action sites and processes, then deductions about the similarity or dissimilarity of the primary actions of different agents from the shapes of their observed dose-response curves are of dubious value. Thus, similar dose-response curves do not, in fact, guarantee similar primary modes of action. For example, all simple exponential dose-response curves are similar (i.e., superimposable by scaling the dose-axis), but they may be produced by agents with very different mechanisms of action, such as ionising radiation (4, 162), ultraviolet light (532), heat (120, 121), photodynamic action (253), DNA cross-linking agents (128, 434, 453, 454), zinc salts (60), pentachlorophenol (60), and hydrogen peroxide (322). Indeed, Finney (176, 177) drew particular attention to the case in which independently acting agents (i.e., with entirely distinct modes of action) had identical probit regression lines.

On the other hand, dissimilar dose-response curves do not guarantee dissimilar modes of action. Two drugs with wholly similar primary modes of action but with different molecular structures, and therefore different physicochemical properties, may be subjected to different metabolic processes, (including activation and inactivation), may have different rates and/or modes of excretion, may be subject to different restrictions in reaching their common site(s) of action, or may be carried there by different transport mechanisms, so that their dose-response curves may differ substantially. In fact, it was the observation that structurally analogous (even isomeric) insecticides believed to act similarly nevertheless had dose-response curves of markedly different slopes when probit mortality was plotted against log dose (249, 346, 487) that led Hewlett and Plackett to modify the original formulation of Bliss (251, 252, 406). Thus, drugs cannot with confidence be classified as acting similarly or dissimilarly simply on the basis of the shapes of their dose-response curves.

These objections notwithstanding, these ideas have had great influence and, in consequence, many investigators look upon concentration-addition and response-addition as different but entirely equivalent models, perhaps differing in their purpose or the sorts of data to which they may be applied, but otherwise of equal standing and validity (8, 30, 115, 302, 303, 306, 509, 533). This is not the case, and it should therefore be stressed that the former is the general model for zero interaction whereas the latter describes only the restricted class of statistically independent actions. These may be zero-interactive if the dose-response curves are appropriate, i.e., simply exponential, but otherwise they show synergy, zero interaction, or antagonism as the case may be, depending on the forms of the dose-response curves and the particular combination used.

IX. Some Important Aspects of Interactions

A. Significance of Minor Interactions

The biological significance of relatively small deviations from zero interaction requires consideration. This problem principally exercises microbiologists, who com-

UNITED THERAPEUTICS CORP., EX1034, page 30
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

monly disregard evidence of synergy unless the interaction index is less than 0.5 (or sometimes 0.75 (400)) and evidence of antagonism unless it exceeds some number greater than 1 (values selected are arbitrary and variable, and include 1.3 (225), 2 (3, 240, 400, 503), 2.75 (2), 4 (14, 114, 152, 240), and even 8 (373). There are two reasons for this conservatism. One is the large error inherent in microbiological assays, stemming from the custom of using doubling dilutions of agents. The other is a doubt as to whether small interactions are clinically significant.

Errors of measurement can, of course, be reduced by improved techniques, for instance, using dilutions less than 2-fold (41, 260) or continuous dilution (138). Moreover, even small degrees of synergy or antagonism can be distinguished from zero interaction with high statistical confidence by comparison with sham combinations of an agent with itself (223, 541). That minor interactions may be significant in vivo is shown by considering pharmacokinetic factors. A relatively small degree of bowing of the isobole towards or away from the zero interaction line may make a considerable difference to the duration of effective drug levels in vivo (fig. 19), and should therefore not be regarded as being therapeutically unimportant (45).

B. Agent multiplicity

It is important to know whether simply increasing the number of agents in a combination affects the level or type of interaction produced. The most clear-cut results are for combinations of antibiotics, where measurements are made in relatively simple and reproducible *in vitro* systems. For example, Armstrong (17) tested combinations of two or three antibiotics against various isolates of *Pseudomonas aeroginosa* and *Proteus vulgaris*. With combinations of two drugs, 70% of tests showed synergy and 25% antagonism whereas, with triple combinations, all tests showed synergy. With both organisms, the highest level of synergy was shown by triple combinations. Similarly, Berenbaum et al. (50) compared the effects of double and triple combinations on *Pseudomonas maltophilia*. With double combinations, 75% were synergistic, compared with 98% of triple combinations. Again, the highest level of synergy was found with triple combinations. An important finding was that the level of synergy of a combination of two antibiotics was generally increased by adding a third, even if this was one to which the organism was resistant, and this effect operated even when the organism was resistant to all three antibiotics on their own. This finding may have implications for the treatment of infections caused by multiply resistant organisms. (See also ref 152 for anti-malarial drugs.) Yu et al. (560) carried out similar studies with *Staphylococcus epidermidis* and found that 20% of double combinations were synergistic while 75% showed antagonism. With triple combinations, the corresponding proportions were 60% and 40%. Finally, Odds (388) compared double, triple, and quadruple combinations of antifungal agents. The proportions showing synergy were, for combinations of two, three, and four, respectively, 47%, 57%, and 91%, and the proportions showing antagonism were 36%, 34%, and 0.

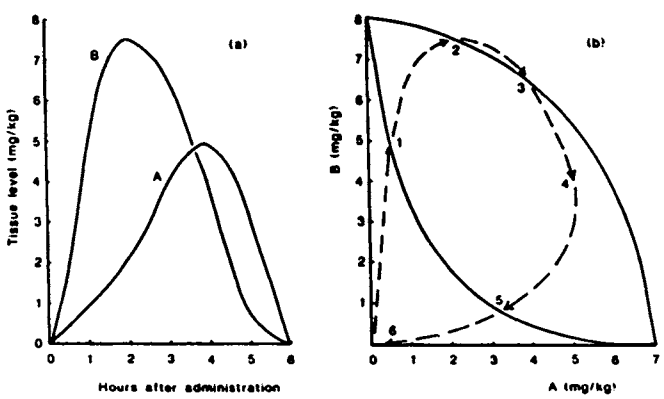

FIG. 19. (*a*) Curves showing the variation with time after administration in the concentrations at the site of action of drugs *A* and *B*. From these curves, the joint concentrations of the agents at specified times (up to 6 hr after administration) may be read. These are indicated by the arrowed path in (b), which also shows isoboles for the concentrations that produce some required effect of *A* and *B*. If the isobole shows moderate synergy, effective levels are reached at 1 hr after administration and persist until 5 hr. On the other hand, if the isobole shows moderate antagonism, the required levels are maintained only between 2 hr and 3 hr after administration. (With a slightly more antagonistic isobole, the required levels would not have been reached at any time).

It would be premature to draw general conclusions from these few examples, but they strongly suggest that in many cases the likelihood of occurence of synergistic reactions and the degree of synergy may both increase with the number of agents in the combination. The effects of agent multiplicity clearly require further examination in a variety of systems, and an economical strategy for examining interactions in multi-agent combinations has been described (50).

X. Therapeutic Synergy. Multifunctional Interactions

So far in this review, only interactions between agents in respect of single effects (monofunctional interactions) have been examined. However, all agents have multiple effects at the level of whole cells or individuals and, while it is sometimes the case that one effect is of such overwhelming importance that others may virtually be ignored, this is not true for most biologically active agents. For example, many cancer chemotherapeutic agents have such pronounced toxicity for normal tissues that therapeutic options are governed at least as much by these effects as by the desired anti-tumour effect.

Accordingly, we have to consider the phenomena generated by multi-functional interactions in the same individual. Two examples from the asthma literature will illustrate what is involved.

Wolfe et al. (548) treated asthmatic patients with

UNITED THERAPEUTICS CORP., EX1034, page 31
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

terbutaline (either 2.5 or 5 mg) or aminophylline (either 200 or 400 mg) or with a combination of the two lower doses. Fig. 20 shows changes in the 1-sec forced expiratory volume (FEV_1) and the incidence of side-effects. The isobole for peak rise in FEV_1 is concave-up, indicating synergy, whereas that for the incidence of toxic effects is concave-down, indicating antagonism. Now suppose the maximum acceptable incidence of side-effects is set at 13%. With terbutaline alone, this is reached at a dose between 2.5 and 5 mg, where the increase in FEV_1 is between 16 and 25%. With aminophylline alone, this incidence of toxicity is reached at a dosage of 200 mg, where the increase in FEV_1 is 14%. Thus, the best therapeutic effect that can be achieved with a single drug, within the specified limit on toxic effects, is an increase in FEV_1 of 16 to 25% with terbutaline. Now the same limiting toxicity is produced by the combination of 2.5 mg terbutaline with 200 mg aminophylline, but here the increase in FEV_1 is 32%. Therefore, within a specified toxicity limit, a combination of these drugs enables a better therapeutic effect to be achieved than either drug alone.

The opposite state of affairs is illustrated by the results of Dyson and Campbell (156), who treated asthmatic patients with choline theophylline, salmefamol or a combination of these (fig. 21). The therapeutic effect measured was increase in peak flow and the toxic effect was tremor (the lower the tremor index, the greater the tremor). The isobole for the therapeutic effect shows antagonism (fig. 21a) while that for toxicity shows synergy (fig. 21b). Now suppose that the limit on toxicity is taken as a tremor index of −3.6%. With choline theophylline, this is produced by a dose greater than 800 mg/day, which gives a rise in peak flow of 5%. With salmefamol alone, this level of toxicity is produced by a dose

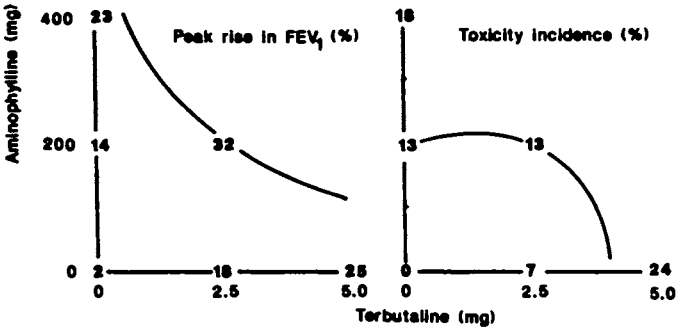

FIG. 20. A greater therapeutic effect may be achieved with a combination of drugs than with either drug alone if therapy is constrained by a specified limit on toxicity and the isoboles for the therapeutic and toxic effects show synergy and antagonism respectively. Data from fig. 1 and table 3 of Wolfe et al. (548) for the effects of terbutaline and aminophylline alone and in combination on FEV_1 and toxicity in asthmatic patients. If the toxicity limit is set at a 13% incidence of side effects, the best that can be achieved with one drug alone within this limit is an increase in FEV_1 of 16–25% with 2.5–5 mg terbutaline. However, within the same toxicity limit, a combination of 2.5 mg terbutaline with 200 mg aminophylline produces an increase of 32% in FEV_1.

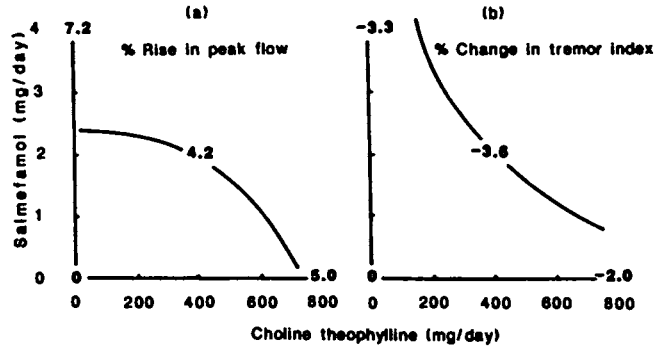

FIG. 21. Combinations of drugs may be therapeutically inferior to single drugs. Data from tables 2 and 4 of Dyson and Campbell (156) for the effects of choline theophylline and salmefamol, alone and in combination, on peak flow and tremor in asthmatic patients. The limiting toxicity is set here as a tremor index of −3.6%. This is produced by a combination of 400 mg/day theophylline with 2 mg/day salmefamol, which gives a 4.2% increase in peak flow. However, larger increases in peak flow (>5.0% and >7.2%) are produced by 800 mg/day theophylline or by 4 mg/day salmefamol on their own, and neither of these cause as much tremor as the combination.

greater than 4 mg/day, which gives a 7.2% rise in peak flow. However, the combination of 400 mg/day theophylline with 2 mg/day salmefamol, which produces the same level of toxicity, gives an increase in peak flow of only 4.2%. Thus, within the specifed toxicity limit, either drug on its own is therapeutically more effective than the combination.

Accordingly, when isoboles for the therapeutic effect show synergy and those for toxicity show antagonism, it is to be expected that the use of combinations will allow therapeutic effects to be achieved that cannot be obtained with the agents on their own without exceeding limits on toxicity. When the reverse state of affairs holds, use of combinations may be positively disadvantageous.

It will be noted that the approach adopted in these examples was first to set a constraint in terms of toxicity and then to determine the maximum therapeutic effect that could be obtained subject to this constraint. In theory, the complementary approach could have been adopted, i.e., to first set a minumum acceptable therapeutic effect and to then determine what toxicity would be incurred in achieving this effect. However, the second approach is not in general practicable because what is set as a minimum acceptable therapeutic effect may turn out to be unachievable with the agents being used, whereas it is always possible to set a level of maximum acceptable toxicity (even if it is decided that the acceptable level is absence of detectable toxicity). In practice, therefore, it is the first approach that is adopted, consciously or by implication, in most clinical investigations of this type.

The two examples discussed above are relatively clear-cut. Therapeutic advantage may however also be obtained in less obvious circumstances, for instance, when the isoboles for therapeutic and toxic effects both show synergy or both show antagonism, or even when the

UNITED THERAPEUTICS CORP., EX1034, page 32
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

isoboles for all relevant effects show zero interaction (41, 46, 48). Exploiting the differences in the interactions for therapeutic and toxic effects is the basis of what is usually termed "therapeutic synergy" (209, 210, 546). The examples discussed above show that this is appropriately defined as a problem in optimisation, that is, given a set of agents (and relevant variables such as dosage, intervals between doses, etc), the problem is to find the combination of variables that produces the greatest therapeutic effect without violating a specified toxicity constraint or set of constraints (42, 85, 90, 527).The view was earlier expressed (42) that "therapeutic synergy" was an unfortunate term in that it confounded two issues that appeared to be essentially different, i.e., (*a*) interactions between agents with respect to single specified effects, combinations being defined unequivocally as synergistic, zero-interactive or antagonistic according to their position with reference to the linear isobole for that effect or the magnitude of the interaction index; and (*b*) the way in which multifunctional interactions could be exploited for therapeutic or other aims (therapeutic synergy). Possibilities for such exploitation might exist whether the individual interactions were synergistic, zero-interactive, or antagonistic and even when there were no interactions in respect of any relevant effect.

While this distinction had the merit of emphasising that differences between interactions for different effects could be exploited even when no individual effect showed synergy, it had the demerit of implying an unnecessary separation between two aspects of the study of interactions. There would be advantages in bringing both aspects of this field into the same conceptual framework, and this may be effected as follows.

The model for monofunctional zero interaction is the sham combination of an agent with itself, which generates a linear isobole for the effect under consideration. Thus, if the isobole for the effect of a combination of different agents is not linear, we know that an interaction is present. The model for multifunctional zero interaction is again the sham combination of an agent with itself, which generates linear and parallel isoboles for all types of effects. These isoboles are similar, i.e., they can all be superimposed on each other by simple linear scalings of the dose-axes. The purpose of examining multiple types of effect is to determine whether the relations between them vary with the combination, for it is only this feature that confers therapeutic or other advantage on one combination as compared with another. This is equivalent to determining whether the isoboles for different types of effect are similar or dissimilar. The convergence between monofunctional and multifunctional interactions is thus brought about by linearizing the isobole for an effect of interest by an appropriate transformation and examining the effects of this on the isoboles for other relevant effects. If the transformation converts all isoboles to parallelism and linearity, we have mimicked the model of the sham combination of an agent with itself (or with dilutions of itself) which is necessarily zero-interactive, and this shows that all combinations show identical relations between the different effects. On the other hand, if the transformation results in nonlinearity of remaining isoboles, then different combinations will differ in the spectrum of effects they produce.

The isobole to be linearized may be, for instance, that for maximum acceptable toxicity or other cost, minimum acceptable therapeutic effect or other gain, or the union of segments of different isoboles that bound a region of acceptable combinations (see ref. 42, figs. 24 and 30). For example, in fig. 22a, agents *A* and *B* show antagonism for both therapeutic and toxic effects. If the isobole for the toxic effect is linearized, that for the therapeutic effect becomes concave-up. If the isobole for the therapeutic effect is linearized, that for the toxic effect is concave-down. Thus we have either "therapeutic synergy" (fig. 22b) or "toxic antagonism" (fig. 22c), which are entirely equivalent in terms of therapeutic aims. This matter will not be pursued further here, but is raised simply to show that conventional synergy and so-called "therapeutic synergy" are not distinct fields of study, unconnected except for a trivial coincidence of terminolgy, but represent respectively the simpler and more complex aspects of the same problem.

XI. Finding the Optimum

The problem of optimisation underlies perhaps most investigations in combination therapy, but has received little formal consideration. The matter is usually approached by means of conventional clinical trials, generally of fairly large scale, in which two (occasionally more) regimens are compared. If one regimen produces a significantly better therapeutic result than the other and is less toxic, or at least not more toxic, then it is obviously the preferred regimen. However, if it produces both greater therapeutic and toxic effects (which is more often the case), the choice is less clear, but usually depends on whether the increased toxicity is thought to be a worthwhile price to pay for the increased therapeutic effect. However, this is merely the start of the investigator's problems for, even when it has convincingly been shown that one regimen is indeed better than the other, the sheer complexity of most multi-drug regimens makes it very difficult or impossible to know with any assurance how to proceed.

For example, in cancer chemotherapy, widely used regimens may include four to five (sometimes up to 10) different agents (61, 70, 81, 92, 143, 184, 209, 210, 214, 460, 546) and, in the treatment cf serious infections, combinations of four to five antibiotics have been used (141, 495, 558). In such regimens, the variables include dose of each agent, intervals between doses, number of doses per course, number of courses, interval between courses and so on, so that a regimen of, say, five agents

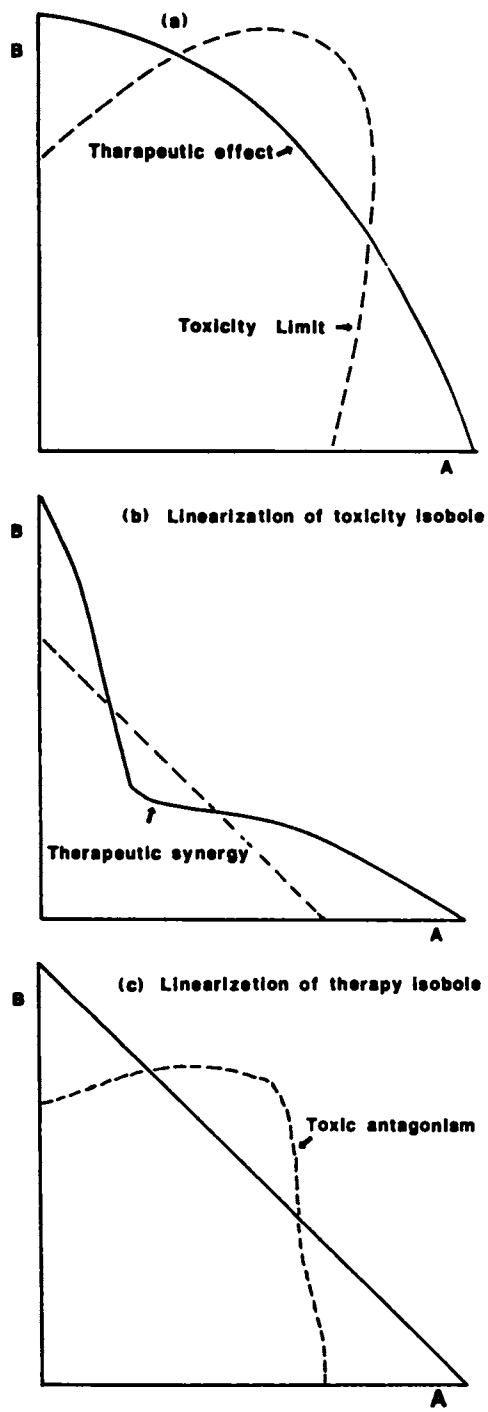

FIG. 22. "Therapeutic synergy" (and the equivalent "toxic antagonism") are multifunctional interactions corresponding respectively to conventional synergy and antagonism, which are monofunctional interactions. (*a*) Agents *A* and *B* show conventional antagonism for both therapeutic and toxic effects. Either isobole may be linearized (for instance, by scaling the coordinates d_a and d_b for each combination on the isobole to $I^{-1}a$ and $I^{-1}d_b$, where I is the interaction index for that combination. (*b*) The transformation that linearizes the toxicity isobole makes the isobole for the therapeutic effect concave-up, i.e., it shows "therapeutic synergy". (*c*) The transformation that linearizes the therapeutic effect isobole leaves the toxicity isobole concave-down, i.e., it shows "toxic antagonism".

may comprise at least 25 independent variables, each of which may profoundly influence therapeutic effect and toxicity. Thus if, in a clinical trial comparing two regimens, i.e., two particular arrangements of these 25 variables, one arrangement were found to produce a significantly better effect than the other arrangement, the question would arise as to how these 25 variables should be changed to produce a still more effective arrangement, and the answer will rarely be obvious.

A naive approach is to find the optimum level of each variable in turn in the expectation that this will give the optimum levels of all. Figure 22 shows why this approach is usually doomed to failure. This figure shows the prolongation of survival in mice with L1210 leukaemia treated with various doses of carminomycin and cyclophosphamide, alone or in combination (24). The optimum dose of carminomycin alone is 1.08 mg/kg, which increases survival by 60%. If that dose is kept fixed and the dose of cyclophosphamide is varied, the best therapeutic effect obtained is an 88% increase in survival (with a combination of 1.08 mg/kg carminomycin and 100 to 120 mg/kg cyclophosphamide). Alternatively, the optimum dose of cyclophosphamide alone is 180 mg/kg and, if this is kept fixed and the dose of carminomycin varied, the best combination is 0.4 mg/kg carminomycin with 180 mg/kg cyclophosphamide, which prolongs survival by 181%. However, a 331% increase in survival could have been obtained with a combination of 0.65 mg/kg carminomycin with 140 mg/kg cyclophosphamide, but this fact could not be discovered using this method of searching for the optimum. It is relevant that Wampler et al. (526) showed that the optimum in a three-variable experiment could not be found if two variables were optimised first and then kept fixed at the levels thus determined while the third variable was subsequently explored.

The difficulty in these cases arises from the fact that the principal axes of the response surface in biological experiments are not generally parallel to the dose-axes (24, 86, 89, 93, 477, 526, 545), and therefore the optimum dose of each agent on its own is not its optimum dose in combination. Accordingly, optimum drug combinations cannot generally be found by exploring one variable, or a limited number of variables, at a time. All relevant variables must be explored together. However, this raises a huge logistical problem. For example, with a combination of five agents, even if every variable except dose were fixed, examining combinations at only three dose-levels would entail comparing 125 different combinations.

Such blunderbuss approaches are not feasible, and more efficient methods must be used. Two possible approaches will be considered here: response surface methods and direct search methods.

A. Response Surface Modelling

Here, the therapeutic effect, or any other effect of interest, is measured for a predetermined number of

different combinations of predetermined composition. The varying levels of observed effect are then fitted approximately by a "response surface," i.e., some algebraic function, the maximum of which (if there is a maximum) is found by standard mathematical methods (65–68, 374). This approach has been explored extensively by Carter and his colleagues (85–93, 198, 375, 472–474, 477, 526, 527, 545). There are two difficulties here. The first, perhaps not very important, because it has a fairly obvious remedy, is the temptation to fit to the data functions that are mathematically easy to handle but which may give a misleading fit. For example, consider the experiments of Solana et al. (474) on the production of sister-chromatid exchanges (SCE) in Chinese hamster cells by combinations of ethylnitrosourea (ENU) and *cis*-diamminedichloroplatinum (II) (DDP). A second-order polynomial was fitted to the DDP response-curve but, as the slope of the curve decreased with increasing dose, the coefficient of the second-order term was necessarily negative. This implied that SCE/cell rose to a maximum and then fell as dosage of DDP was increased further, whereas the experimental data showed that it rose over the whole dose range. The same anomaly was found in the work of Wilson et al. (545), who fitted a second-order polynomial to the results of experiments on combinations of methylmethane sulphonate and ENU. As both second-order terms were negative, the surface showed a peak, with a fall in SCE/cell as the concentrations of either drug was increased further. The experimental data, however, showed only an increase.

A far more important difficulty is that the number of combinations that must be examined in order to fit a second-order surface rises more or less exponentially with the number of variables. For instance, even with the highly economical central composite experimental design of Box and Wilson (68), a problem in n variables entails testing $2^n + 2n + 1$ combinations. Thus, for a set of five agents in which doses, the intervals between them, and the total number of doses were varied (15 variables), the number of combinations to be tested would be 32,799. Even then, as the examples (474, 545) considered above show, there is no assurance that a surface so fitted would be good enough to indicate the optimum. It is therefore not surprising that the application of this approach to pharmacology has so far been limited to experiments involving two or three variables, and it is difficult to see how it could constitute a practicable approach with the much greater number of variables commonly encountered in clinical therapeutics.

B. Direct Search Methods (DSM)

An alternative approach is a step-wise search of the response surface. The problem is analogous to that of searching for the top of a hill in the dark, with the ability to measure the heights of points selected one at a time but without being able to see the hill as a whole. These points are not at predetermined locations; instead, the location of each is selected during the exploration on the basis of the previous measurements. Common sense suggests that if, at each step in this exploration, the next point in the sequence is located in a direction away from the lowest point in the vicinity, we should eventually reach the top. This idea was first formalised by Spendley et al. (476), and the method is illustrated in fig. 24 as a problem in optimising combinations of two drugs, using again the results of Avery and Cruse (24) for effects of combinations of carminomycin and cyclophosphamide on L1210 leukaemia in mice. In this figure, the contours are isoboles for the therapeutic effect, obtained from fig. 23, but the investigator is at the outset unaware of their position or shape. Initially, the effects of three combinations (represented by the vertices of triangle ABC) are measured. Combination A is found to have the least therapeutic effect of the three, so the triangle is reflected through the centre of the line BC joining the remaining two points to give a new combination D, and A is discarded. The lowest effect in the new triangle BCD is produced by combination B, so the triangle is reflected in turn through the centre of line CD to give combination E, and so on. Successively formed triangles climb the response surface, turning with the contours, until one,

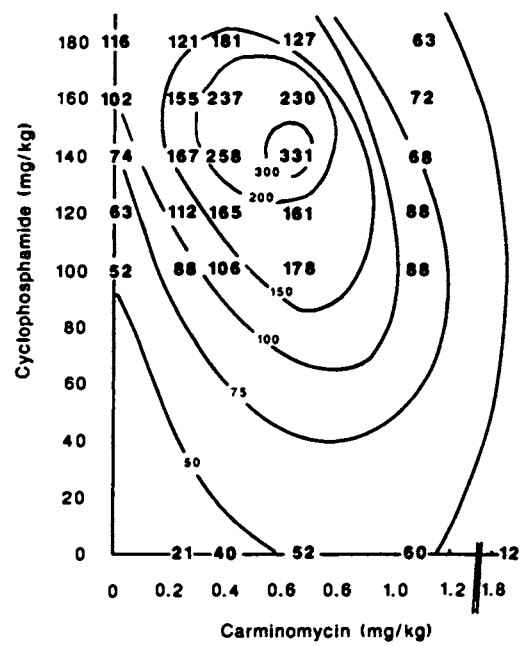

FIG. 23. Isoboles for prolongation of survival in mice with L1210 leukaemia given carminomycin and cyclophosphamide, alone or in combination (data from table 1 of Avery and Cruze (24)). The greatest prolongation in survival with carminomycin alone is 60%, with a dose of 1.08 mg/kg, and the greatest prolongation with cyclophosphamide alone is 116%, at a dosage of 180 mg/kg. Overall, the greatest prolongation, by 331%, is achieved with a combination of 0.65 mg/kg carminomycin with 140 mg/kg cyclophosphamide. The response surface forms a hill, and the isoboles for prolongations in survival of 150–300% form closed curves that touch neither dose-axis. It should be noted that the principal axes of the response surface are not parallel to the dose axes, so that a search for the optimum dose of one agent on its own followed by a similar search for the optimum of the other will not locate the optimum combination.

UNITED THERAPEUTICS CORP., EX1034, page 35
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

KLM, straddles the peak, after which there is no further improvement, successive reflections merely switching between triangles KLM and KMN. The peak may now be approached more closely if desired by reducing the size of the triangles. This approach is readily generalised for n variables, using instead of a triangle a "simplex" of $n+1$ combinations.

One of the most important feature of DSM is its economy. Although in the two-variable problem shown in fig. 24 it was necessary to examine three starting combinations and then to take a further 11 steps to reach the optimum, an analogous problem in n variables merely increases the size of the starting simplex and each subsequent step requires only one new combination. Thus, if 11 steps were needed to reach the optimum, a problem in 15 variables would require a starting simplex of 16 combinations and 11 further steps, a total of 27 combinations compared with the 32,799 required by a response modelling method.

Further, as in response modelling, sample sizes at each point may be quite small (Wampler (526) used only two animals per group in an experiment); there is no need to ensure that the differences between the effects of successive combinations are statistically significant, as it is the overall trend that is important here (476).

Any stepwise search may, of course, fail to locate the optimum of the response surface if this has more than one peak and the search happens to locate a minor peak first. The problem of finding the global optimum in such circumstances much concerns investigators of optimization methods (53) but fortunately it seems unlikely to be of much importance in biological studies, where adequately characterised response surfaces almost always appear to show single peaks (88, 89, 91–93, 198, 472, 477, 478, 545).

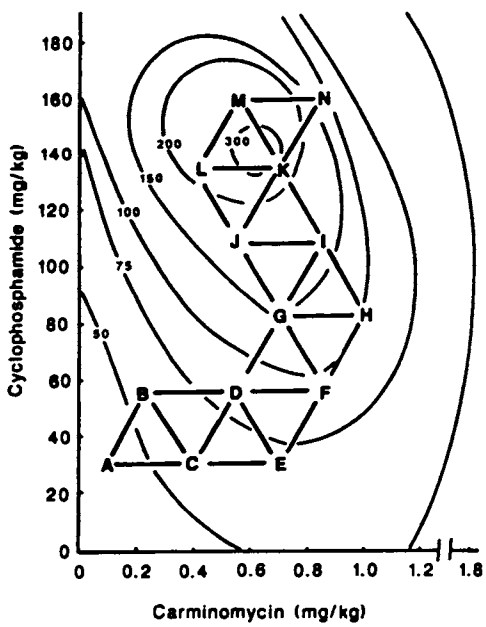

FIG. 24. A direct, stepwise search of the response surface shown in fig. 23, using the method of Spendley et al. (476). The search starts with triangle ABC. The lowest point on the surface here is A. This is reflected through line BC to give point D. The lowest point in the new triangle BCD is B, which is reflected in turn to give E, and so on. Successive triangles climb the surface, the direction of search turning with the contours, until the region of the optimum is reached. If the search is continued without modification at that point, it makes no further progress, but switches back and forth between triangles KLM and KMN. If desired, the search can then be made to converge more closely on the optimum by reducing the size of the triangles.

One drawback of the simplex method of Spendley et al. (476) is that the shape and size of the simplex and its possible directions of movement are rigidly fixed from the outset and do not automatically adapt themselves to the shape of the response surface as it is explored or take account of toxicity constraints. The method was therefore improved by Nelder and Mead (376) who incorporated rules that allowed the simplex to elongate in favourable directions and to contract if a movement was unfavourable. Box (69) introduced the further modification of using a "complex" of $>n+1$ points and allowing the complex to retreat if it violated a constraint.

Even with these improvements, these methods, which were designed primarily for use in mathematical and industrial problems, are not adapted to take into account two important problems that arise in the clinical use of toxic drugs. These are (*a*) violation of constraints means unacceptable toxicity to patients and possibly death, and (*b*) almost all measurements made in a clinical context are subject to large statistical error. We found, in computer simulations, using mathematical problems with known solutions and artificially induced error, that the Nelder–Mead and Box methods could not cope with error of the magnitude typical of biological experiments, in which standard errors may easily be 0.2 to 0.3 of the mean, or more. The reason for failure was that the direction of each move depended too much on the location of the single combination discarded at that step. When error is high, there is a high probability that the wrong combination will be selected, acting in effect like a misleading signpost, and that this wrong choice misdirects the next move.

Accordingly, the methods have been modified in attempts to overcome these difficulties. The main, and most effective, modification was to remove the undue weight attached to the discarded point. The combinations in each successively formed complex were partitioned between the best and worst halves and the direction of the next move was set along the line joining the mean positions of these two sets. Thus, if $\mathbf{x}_b$ and $\mathbf{x}_w$ are the mean values of any variable in the best and worst sets, respectively, its value in the new combination is $(\alpha + 1)\mathbf{x}_b - \alpha\mathbf{x}_w$, where α is some positive number (Box (69) recommends putting $\alpha = 1.3$).

The results of a three-variable search with the Nelder–Mead and Box method and with the new method (which, for brevity, will be termed the partition method) are shown in figs. 25 and 26. In these experiments (48a),

UNITED THERAPEUTICS CORP., EX1034, page 36
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

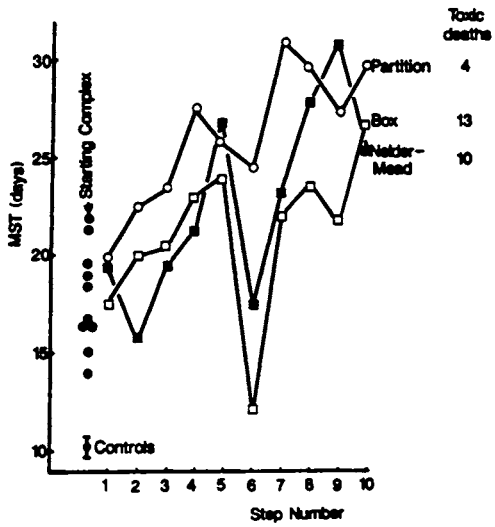

FIG. 25. Effects of combinations of isophosphamide and *N*-acetylcysteine with a varying time interval between the two drugs in mice with advanced L1210 leukaemia. Mean survival time (MST) for groups of eight mice are shown. Drugs were injected once on day 7 of the disease, and the MST of untreated mice was about 10 days. The starting complex consisted of 10 arbitrarily selected combinations of the variables, and gave MSTs of 14–22 days. Ten subsequent steps were then taken using either the Nelder-Mead, Box, or partition search methods (see text). The total number of deaths from drug toxicity during the 10 steps of the search (80 mice) is indicated. Note the large swings in MST with the Nelder-Mead and Box methods, due to excessive drug toxicity, and the more consistent progress and lower overall toxicity of the partition method.

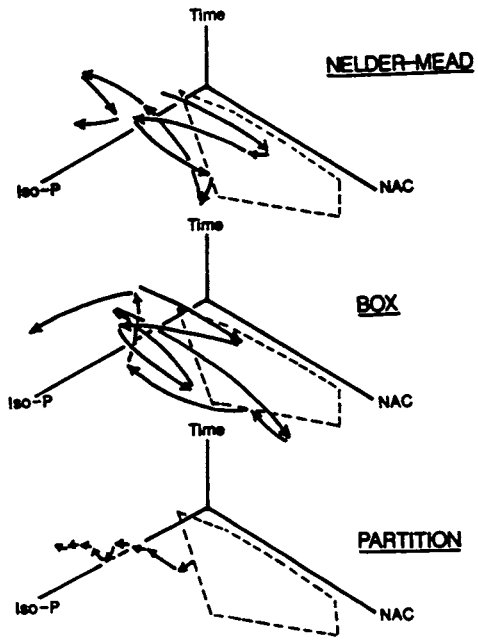

FIG. 26. Paths of the three searches shown in fig. 25 for combinations of isophosphamide (Iso-P), *N*-acetylcysteine (NAC) and varying time interval. The areas enclosed by the dashed lines include the combinations of the starting complex. Note the highly erratic search paths of the Nelder-Mead and Box methods and the highly consistent search path of the partition method.

submitted for publication), mice were injected with L1210 leukaemia cells subcutaneously. This produces a systemic disease that kills usually in 10 to 11 days. Drugs were given on a single occasion on day 7 of the disease, so that the procedure is a stringent test of their efficacy. Clearly, the search paths of the two standard methods are highly erratic and, although combinations with high therapeutic effect are found, there appears to be a large element of chance in this (even a completely random search will occasionally find good combinations). In contrast, the partition method appears to have a much more consistent search path. Overall, it finds more effective combinations and with less cost in toxicity (the latter feature may be due to the lack of highly erratic changes in path, that is, lack of erratic changes in dosage of any of the agents).

The partition method must be regarded as only an interim improvement and undoubtedly requires further investigation and modification. It is important to note that, although this experiment involved only three variables, this restriction was made in order to facilitate visualisation of the search path so that problems in the method could be analysed. There seems to be no reason, when these methods have been further modified to make them more suitable for biological work, why they should not be applied to problems with high numbers of variables.

The advantage of direct search methods are clear.

(*a*) They constitute the only known practicable approach to handling problems with numerous variables, and they avoid the pitfalls associated with exploring one variable (or a limited number of variables) at a time.

(*b*) They entail no restrictive assumptions about the shape of the response surface, and therefore avoid the problems that arise from the inevitable divergence between real and mathematically fitted surfaces.

(*c*) They are made one step at a time (i.e., only one new combination is examined at each step) so that, in the long run, they are highly economical.

(*d*) Their flexibility is such that new variables may be added and even the rules of the search may be changed after the search has begun. For instance, if a direct search had been restricted to one variable in the problem illustrated in fig. 23 (say, dosage of carminomycin), it would have located a dose of about 1.08 mg/kg as the optimum. If at that stage dosage of cyclophosphamide had been added as a new variable, so that further exploration was of both drugs simultaneously, a direct search method would have little difficulty in ascertaining the correct direction of move towards the optimum dosage of both. This flexibility would be an important consideration in any step-wise search in a clinical context, where irresistible pressure to make changes, including the incorporation of new agents, may arise during the course of a trial of any considerable duration.

XII. Finding the Minimum. Complex Environmental Mixtures

Human beings (and other living organisms) are exposed environmentally to very large numbers of materials of known or possible toxicity. Such materials often occur together in complex mixtures of dozens to many hundreds of constituents. Typical complex mixtures are tobacco smoke, ground-water contaminated with pesticides and herbicides in areas of intensive farming or with industrial chemicals near waste dumps, food containing additives and naturally occurring toxins, and products of combustion. Even when individual toxicants are present in negligible amounts, several may interact together in producing effects. The opportunity for such interactions increases enormously as the number of substances present in a mixture rises and, as shown in section IX B, the degree of interaction between toxicants may also increase with their number. All these facts are matters for considerable concern (101, 125, 199, 323a, 358, 483, 554, 555).

Careful investigations of complex environmental mixtures, such as a representative mix of 25 organic and inorganic chemicals found in ground-water or material leached from a dump site have shown them to have toxic effects (for example depression of immunological functions and teratogenic effects), when administered to laboratory rodents (199, 455b, 456), and no doubt investigation of other typical mixtures in animals will also show toxicity that would be unacceptable if it occurred in humans.

Thus, measures are required to reduce toxicity due to environmental exposure to such mixtures. One obvious recourse is a blanket reduction in the environmental level of the whole mixture, but this option may be practicable in special cases only, such as that of cigarette smoke. However, it has been suggested that, if the agents in a mixture act synergistically, then removing one might produce a disproportionate reduction in effect (147, 447). The possibility should therefore be considered that in some circumstances it might be a more effective use of resources to reduce the level of one or a few key constituents in a mixture that contribute disproportionately to the synergistic interaction, or possibly to eliminate these entirely, rather than attempt a uniform, smaller, and possibly less effective reduction in all constituents.

For example, if it could be shown that a substantial reduction in the level of one or two particular pesticides in contaminated ground-water would disproportionately reduce its toxicity, this might constitute a powerful argument for legislation against their use (even if it might take a long time for such a ban to affect ground-water levels significantly).

The direct search method illustrated in fig. 24, or one of the more recent versions, might constitute a useful strategy for tackling this problem, with the obvious modification that the aim of the search is to find combinations of reducing toxicity instead of increasing therapeutic effect. The search is thus, as it were, run in reverse. Of course, the combination with the absolute minimum toxicity is that which contains no toxic materials at all, and locating this is not the aim of the exercise. The aim is rather to use DSM to find which components of the mixture should be reduced or eliminated so as to reduce toxicity most effectively. An appropriate procedure may be illustrated by reference to fig. 24, supposing the isoboles to represent, not increases in MST, but levels of toxicity. It should first be pointed out that, unlike the surface in this figure, toxicity response surfaces are not likely to peak and then fall with increasing levels of toxicants; they are likely only to increase, and therefore it is only the rising part of this surface that is relevant here (i.e., the bottom left quadrant of the figure).

The toxic mixture with which we are faced at the start of the search may therefore be represented by the combination of 0.7 mg/kg carminomycin with 120 mg/kg cyclophosphamide, with a toxicity level of about 200. The search starts with triangle IJK. The most toxic combination here is K, so this is discarded and reflection through the line JI gives combination G. The most toxic combination in the new triangle is J, the next reflection gives H, and so on. Thus, the search begins with a progressive reduction in the level of cyclophosphamide, and this reduces toxicity levels from 200 to 75. Subsequently, the level of carminomycin is reduced, and this lowers toxicity levels further, from 75 to 50. Evidently, faced with the problem of reducing the level of toxicity of the starting mixture by reducing the level of one component, the search course would indicate that the one to reduce was cyclophosphamide.

This type of strategy might be applied, for instance, to representative mixtures of toxicants, the composition of which could be varied at will (103, 199, 554, 555). It may not be necessary, using DSM, to start with combinations of all toxicants known to be present because, as explained above, the methods are highly flexible and it might be feasible to start with mixtures of fewer components, perhaps those thought to be the most important, and to add others subsequently. However, the efficiency of such modifications requires investigation.

Constraints may be placed on the search if relevant. For instance, in the context of reducing the toxicity of environmental mixtures, cost is an important factor and might appropriately be set as a constraint.

The advantages of DSM in such problems of minimization are the same as those they have in problems of therapeutic optimization, i.e., they are economical of resources and highly adaptable, they entail no assumptions about the underlying response surface, and they appear to represent the only feasible approach to problems of such complexity.

Acknowledgments. I am grateful to the Cancer Research Campaign for support, and to Ting-Chao Chou, Peter Gessner, William Greco, Ralph Kodell, Gerald Pöch, and Hans Dieter Unkelbach for stimulating

UNITED THERAPEUTICS CORP., EX1034, page 38
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

and helpful discussion and correspondence; to Colleen Kelly, Ralph Kodell, and Raymond Wang for making unpublished manuscripts available; and to Dr. G. Verriopoulos for providing the laboratory data used for constructing fig. 12.

REFERENCES

1. Abou-Issa, H., Koolemans-Beynen, A., Minton, J. P., and Webb, T. E.: Synergistic interaction between 13-*cis*-retinoic acid and glucarate: Activity against rat mammary tumor induction and MCF-7 cells. Biochem. Biophys. Res. Commun. **163:** 1364–1369, 1989.

1a. Ackland, S. P., Schilsky, R L., Beckett, M. A., and Weichselbaum, R. R.: Synergistic cytotoxicity and DNA strand break formation by bromodeoxyuridine and bleomycin in human tumor cells. Cancer Res. **48:** 4244–4249, 1988.

2. Ahmet, Z., and Houang, E.: Synergy study of carumonam and ceftazidime with aminoglycosides. J. Antimicrob. Chemother. **20:** 288–290, 1987.

3. Al-Nakib, W., and Tyrrell, D. A. J.: A "new" generation of more potent synthetic antirhinovirus compounds: comparison of their MIC's and their synergistic interactions. Antiviral Res. **8:** 179–188, 1987.

4. Alper, T.: The cell as a single-hit detector. Br. J. Cancer: Suppl VIII **58:** 32–38, 1987.

5. Altin, J. G., and Bygrave, F. L.: Second messengers and the regulation of Ca^{2+} fluxes by Ca^{2+}-mobilizing agonists in rat liver. Biol. Rev. **63:** 551–611, 1988.

6. Amemiya, H., Suzuki, S., Niiya, S., Watanabe, H., and Kotake, T.: Synergistic effect of cyclosporine and mizoribine on survival of dog renal allografts. Transplantation **46:** 768–771, 1988.

7. Anderson, E. T., Young, L. S., and Hewitt, W. L.: Antimicrobial synergism in the therapy of gram-negative rod bacteremia. Chemotherapy **24:** 45–54, 1978.

8. Anderson, P. D., and Weber, L. J.: The toxicity to aquatic populations of mixtures containing certain heavy metals. *In* Proc. International Conference on Heavy Metals in the Environment, pp. 933–953, October 27–31, 1975, Toronto. Canada Institute for Scientific and Technical Information, 1976.

9. Ando, Y., Jacobson, H. R., and Breyer, M. D.: Phorbol ester and A23187 have additive but mechanistically separate effects on vasopressin action in rabbit collecting tubules. J. Clin. Invest. **81:** 1578–1584, 1988.

10. Ankenbauer, W., Strähle, U., and Schütz, G.: Synergistic action of glucocorticoid and estradiol responsive elements. Proc. Nat. Acad. Sci. **85:** 7526–7530, 1988.

11. Anzano, M. A., Roberts, A. B., Meyers, C. A., Komoriya, A., Lamb, L. C., Smith, J. M., and Sporn, M. B.: Synergistic interaction of two classes of transforming growth factors from murine sarcoma cells. Cancer Res. **42:** 4776–4778, 1982.

12. Archer, C. B., Frohlich, W., Page, C. P., Paul, W., Morley, J., and MacDonald, D. M.: Synergistic interactions between prostaglandins and Paf-acether in experimental animals and man. Prostaglandins **27:** 495–501, 1984.

13. Archer, C. B., Page, C. P., Juhlin, L., Morley, J., and MacDonald, D. M.: Delayed-onset synergism between leukotriene B4 and prostaglandin E2 in human skin. Prostaglandins **73:** 799–805, 1987.

14. Arditi, M. and Yogev, A.: In vitro interaction between rifampin and clindamycin against pathogenic coagulase-negative staphylococci. Antimicrob. Agents Chemother. **33:** 245–247, 1989.

15. Ardlie, N. G., Bell, L. K. and McGuiness, J. A.: Synergistic potentiation by epinephrine of collagen or thrombin-induced calcium mobilization in human platelets. Thromb. Res. **46:** 519–526, 1987.

16. Ariëns, E. J., Van Rossum, J. M., and Simonis, A. M.: A theoretical basis of molecular pharmacology. Part 1. Interactions of one or two compounds with one receptor system. Arzneimittelforschung **6:** 282–293, 1956.

17. Armstrong, C. W. J., and Larner, A. E.: The effect of combinations of antibiotics on *Pseudomonas aeroginosa* and on *Proteus vulgaris*, in vitro and in vivo. J. Lab. Clin. Med. **37:** 584–592, 1951.

18. Ashford, J. R.: Quantal responses to mixtures of poisons under conditions of simple similar action—the analysis of uncontrolled data. Biometrika **8:** 74–88, 1958.

19. Ashford, J. R.: General models for the joint action of mixtures of drugs. Biometrics **37:** 457–474, 1981.

20. Ashford, J. R., and Cobby, J. M.: A system of models for the action of drugs applied singly or jointly to biological organisms. Biometrics **30:** 11–31, 1974.

21. Ashford, J. R., and Smith C. S.: General models for quantal response to the joint action of a mixture of drugs. Biometrika **51:** 413–428, 1964.

22. Ashford, J. R., and Smith, C. S.: Models for the non-interactive joint action of a mixture of stimuli in biological assay. Biometrika **53:** 49–59, 1966.

23. Auerbach, C.: Mutation Research. Problems, Results and Perspectives. Chapman and Hall, London, 1976.

24. Avery, T. L., and Cruze, P. G.: Therapeutic performance of carminomycin- cyclophosphamide against L1210 leukemia. Cancer Res. **38:** 2892–2825, 1978.

25. Ayisi, N. K., Gupta, V. S., Meldrum, J. B., Taneja, A. K., and Babiuk, L. A.: Combination therapy: interaction of 5-methoxymethyldeoxyuridine with adenine arabinoside, 5-ethyldeoxyuridine, 5-iododeoxyuridine, and phosphonacetic acid against herpes simplex virus types 1 and 2. Antimicrob. Agents Chemother. **17:** 558–566, 1980.

25a. Babich, M. A., Devanas, M. A., and Stotzky, G.: The mediation of mutagenicity and clastogenicity of heavy metals by physicochemical factors. Environ. Res. **37:** 253–286, 1985.

26. Babich, M. A., and Day, A. S., III: Synergistic killing of virus- transformed cells with interferon and *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine. Carcinogenesis **10:** 265–268, 1989.

27. Bachelet, M., Adolfs, M. J. P., Masliah, J., Bereziat, G., Vargaftig, B. B., and Bonta, I. L.: Interaction between PAF-acether and drugs that stimulate cyclic AMP in guinea-pig alveolar macrophages. Eur. J. Pharmacol. **149:** 73–78, 1988.

28. Badwey, J. A., Robinson, J. M., Horn, W., Soberman, R. J., Karnovsky, M. J., and Karnovsky, M. L.: Synergistic stimulation of neutrophils. Possible involvement of 5-hydroxy-6,8,11,14-eicosatetraenoate in superoxide release. J. Biol. Chem **263:** 2779–2786, 1988.

29. Bahnson, R. R., and Ratliff, T. L.: Inhibition of mouse bladder tumor proliferation by alpha difluoromethylornithine and interferon in vitro and in vivo. J. Urol. **139:** 1367–1371, 1988.

30. Ball, W. L.: The toxicological basis of threshold limit values: 4. Theoretical approach to prediction of toxicity of mixtures. Am. Ind. Hyg. Assoc. J., **20:** 357–363, 1959.

31. Barnes, N. C., Watson, A., Koulouris, N., Piper, P. J., and Costello, J.: Effect of preinhalation of leukotriene D4 on sensitivity to inhaled prostaglandin F2 Thorax **39:** 697, 1984.

32. Barnes, P. J., Chung, K. F., and Page, C. P.: Inflammatory mediators and asthma. Pharmacol. Rev. **40:** 49–84, 1988.

33. Basran, G. S., Morley, J., Paul, W., and Turner-Warwick, M.: Evidence in man for synergistic interactions between putative mediators of acute inflammation and asthma. Lancet **1:** 935–937, 1982.

34. Bény, J.-L., and Baertschi, A.-J.: Synthetic corticoliberin needs arginine vasopressin for full corticotropin releasing activity. Experientia **38:** 1078–1079, 1982.

35. Benz, C., Cadman, E., Gwin, J., Wu, T., Amara, J., Eisenfeld, A., and Dannies, P.: Tamoxifen and 5-fluorouracil in breast cancer: cytotoxic synergism *in vitro*. Cancer Res. **43:** 5298–5303, 1983.

36. Beran, M., McCredie, K. B., Keating, M. J., and Gutterman, J. U.: Antileukemic effect of recombinant tumor necrosis factor α in vitro and its modulation by α and γ interferons. Blood **72:** 728–738, 1988.

37. Berenbaum, M. C.: Dose-response curves for agents that impair cell reproductive integrity. A fundamental difference between dose-response curves for antimetabolites and those for radiation and alkylating agents. Br. J. Cancer **23:** 426–433, 1969.

38. Berenbaum, M. C.: Immunosuppression by platinum diamines. Br. J. Cancer **25:** 208–211, 1971.

39. Berenbaum, M. C.: Synergy, additivism and antagonism in immunosuppression. A critical review. Clin. Exp. Immunol. **28:** 1–18, 1977.

40. Berenbaum, M. C.: A method for testing for synergy with any number of agents. J. Infect. Dis. **137:** 122–130, 1978.

41. Berenbaum, M. C.: Correlations between methods for measurement of synergy. J. Infect. Dis. **142:** 426–428, 1980.

42. Berenbaum, M. C.: Criteria for analysing interactions between biologically active agents. Adv. Cancer Res. **35:** 269–335, 1981.

43. Berenbaum, M. C.: Consequences of synergy between environmental carcinogens. Environ. Res. **38:** 310–318, 1985.

44. Berenbaum, M. C.: The expected effect of a combination of agents: the general solution. J. Theor. Biol. **114:** 413–431, 1985.

45. Berenbaum, M. C.: Minor synergy and antagonism may be clinically important. J. Antimicrob. Chemother. **19:** 271–273, 1987.

46. Berenbaum, M. C.: Synergy and antagonism are not synonymous with therapeutic advantage and disadvantage. J. Antimicrob. Chemother. **21:** 497–500, 1988.

47. Berenbaum, M. C.: Isobolographic, algebraic and search methods in the analysis of multi-agent synergy. J. Am. Coll. Toxicol. **7:** 927–938, 1988.

48. Berenbaum, M. C. Synergy in the pathogeneisis and treatment of asthma. *In* Asthma Reviews, ed. by J. Morley, Academic Press, London, pp. 27–74, 1989.

48a. Berenbaum, M. E.: Direct search methods in the optimisation of cancer chemotherapy regimens. Br. J. Cancer, in press, 1990.

49. Berenbaum, M. C., Cope, W. A., and Bundick, R. V.: Synergistic effect of cortisol and prostaglandin E_2 on the PHA response. Relation to immunosuppression induced by trauma. Clin. Exp. Immunol. **26:** 534–541, 1976.

50. Berenbaum, M. C., Yu, V. L., and Felegie, T. P.: Synergy with double and triple antibiotic combinations compared. J. Antimicrob. Chemother. **12:** 555–563, 1983.

51. Berg, O. G., and Blomberg, C.: Mass action relations *in vivo* with application to the *Lac* operon. J. Theoret. Biol. **67:** 523–533, 1977.

52. Berger, M. R., Schmähl, D., and Zerban, H.: Combination experiments with low doses of three genotoxic *N*-nitrosamines with similar organotropic carcinogenicity in rats. Carcinogenesis **8:** 1635–1643, 1987.

UNITED THERAPEUTICS CORP., EX1034, page 39
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

53. Beveridge, G. S. G., and Schechter, R. S.: Optimization: Theory and Practice. McGraw-Hill, Kogakusha Ltd., 1970.
54. Beverley, J. K. A., and Fry, B. A.: Sulphadimidine, pyrimethamine and dapsone in the treatment of toxoplasmosis in mice. Br. J. Pharmacol. 12: 189–193, 1957.
55. Bich-Thuy, L. T., and Fauci, A. S.: Recombinant interleukin 2 and gamma-interferon act synergistically on distinct steps of in vitro terminal B cell maturation. J. Clin. Invest. 77: 1173–1179, 1986.
56. Bliss, C. I.: The toxicity of poisons appled jointly. Ann. Appl. Biol. 26: 585–615, 1939.
57. Blot, W. J., and Day, N. E.: Synergism and interaction: are they equivalent? Am. J. Epidemiol. 110: 99–100, 1979.
58. Blot, W. J., McLaughlin, J. K., Winn, D. M., Austin, D. F., Greenberg, R. S., Preston-Martin, S., Bernstein, L., Schoenberg, J. B., Stemhagen, A., and Fraumeni, J. F., Jr.: Smoking and drinking in relation to oral and pharyngeal cancer. Cancer Res. 48: 3282–3287, 1988.
59. Boettcher, F. A., Henderson, D., Gratton, M. A., Danielson, R. W., and Byrne, C. D.: Synergistic interactions of noise and other ototraumatic agents. Ear and Hearing 8: 192–212, 1987.
60. Bois, F., Vaillant, M., and Vasseur, P.: Multiple regression analysis of toxic interactions: application to the Microtox test and general comments. Bull. Environ. Contam. Toxicol. 36: 707–714, 1986.
61. Bonadonna, G.: Chemotherapy of malignant lymphomas. Semin. Oncol. Suppl. 6, 12: 1-14, 1985.
62. Borda, I. T., Slone, D., and Jick, H.: Assessment of adverse reactions within a drug surveillance program. J. Am. Med. Assoc. 205: 645–647, 1968.
63. Bouakka, M., LeGendre, P., Jouis, V., Langris, M., Béliard, A., Loyau, G., and Bocquet, J.: Calcium ionophore and phorbol myristate acetate synergistically inhibited proteoglycan biosynthesis in articular chondrocytes by prostaglandin independent mechanism. Biochem. Biophys. Res. Comm. 153: 690–698, 1988.
64. Bounias, M.: Algebraic potential of the Hill equation as an alternative tool for plotting dose (or time)/effects relationships in toxicology: a theoretical study. Fundam. Clin. Pharmacol. 3: 1–9, 1989.
65. Box, G. E. P.: The exploration and exploitation of response surfaces: some general considerations and examples. Biometrics 10: 16–60, 1954.
65a. Box, G. E. P.: Evolutionary operation: a method for increasing industrial productivity. Appl. Stat. 6: 81–101, 1957.
66. Box, G. E. P., and Draper, N. R.: Empirical Model-Building. John Wiley, New York, 1987.
68. Box, G. E. P., and Wilson, K. W.: On the experimental attainment of optimum conditions. J. Roy. Stat. Soc. Series B 13: 1–45, 1951.
69. Box, M. J.: A new method for constrained optimization and a comparison with other methods. Computer J. 8: 42–52, 1965.
70. Bregman, M. D., and Meyskens, F.L., Jr.: Difluoromethylornithine enhances inhibition of melanoma cell growth in soft agar by dexamethasone, clone A interferon and retinoic acid. Int. J. Cancer 37: 101–107, 1986.
71. Bregman, M. D., Peters, E., Sander, D., and Meyskens, F. L., Jr.: Dexamethasone, prostaglandin A, and retinoic acid modulation of murine and human melanoma cells grown on soft agar. J. Natl. Cancer Inst. 71: 927–932, 1983.
71a. Brennan, J. F., and Jastreboff, P. J.: Interaction of salicylate and noise results in mortality of rats. Experientia 45: 731–734, 1989..
72. Brenner-Gati, L., Berg, K. A., and Gershengorn, M. C.: Thyroid-stimulating hormone and insulin-like growth factor-I synergize to elevate 1,2-diacylglycerol in rat thyroid cells. J. Clin. Invest. 82: 1144–1148, 1988.
73. Britton, J. R., Burney, P. G. J., Chinn, S., Papacosta, A. O., and Tattersfield, A. E.: The relation between change in airway reactivity and change in respiratory symptoms and medication in a community study. Am. Rev. Respir. Dis. 138: 530–534, 1988.
74. Brooks, G., Tudor Evans, A., Aitken, A., and Evans, F. J.: Tumour-promoting and hyperplastic effects of phorbol and daphnane esters in CD-1 mouse skin and a synergistic effect of calcium ionophore with the non-promoting activator of protein kinase C, sapinotoxin A. Carcinogenesis 10: 283–288, 1989.
75. Bross, I. D.: Re: "Case-control study of lung cancer in civilian employees at the Portsmouth Naval Shipyard, Kittery, Maine." Am. J. Epidemiol. 127: 202–204, 1988.
76. Brown, K. D., and Holley, R. W.: Insulin-like synergistic stimulation of DNA synthesis in Swiss 3T3 cells by the BSC-1 cell-derived growth inhibitor related to transforming factor type β. Proc. Natl. Acad. Sci. 84: 3743–3747, 1987.
77. Broxmeyer, H. E., Cooper, S., Aubin, B. Y., and Taylor, M. W.: The synergistic influence of human interferon-γ and interferon-α on suppression of hematopoietic progenitor cells is additive with the enhanced sensitivity of these cells to inhibition with interferons at low oxygen tension in vitro. J. Immunol. 135: 2502–2506, 1985.
78. Brunda, M. J., Tarnowski, D., and Davatelis, V.: Interaction of recombinant interferons with recombinant interleukin-2: differential effects on natural killer cell activity and interleukin-2-activated killer cells. Int. J. Cancer 37: 787–793, 1986.
79. Brunden, M. N., Vidmar, T. J., and McKean, J. W.: Drug Interaction and Lethality Analysis. CRC Press, Boca Raton, FL, 1988.
80. Bayson, Y. J., and Kronenberg, L. H.: Combined antiviral effects of interferon, adenine arabinoside, hypoxanthine arabinoside, and adenine arabinoside-5′-monophosphate in human fibroblast cultures. Antimicrob. Agents Chemother. 11: 299–306, 1977.
81. Bunn, P. A., Jr., Cohen, M. K., Ihde, D. C., Fossieck, B. E., Jr., Matthews, M. J., and Minna, J. D.: Advances in small cell bronchogenic carcinoma. Cancer Treat. Rep. 61: 333–342, 1977.
82. Callard, A. E.: Cytokine regulation of B-cell growth and differentiation. Br. Med. Bull. 45: 371–388, 1989.
83. Caplan, A. A., and Su, J. Y.: Interaction of halothane and verapamil in isolated papillary muscle. Anesth. Anal. 65: 463–468, 1986.
84. Caracciolo, D., Clark, S. C., and Rovera, G.: Human interleukin-6 supports granulocyte differentiation of hemopoietic progenitor cells and acts synergistically with GM-CSF. Blood 73: 666–670, 1989.
85. Carter, W. H., Jr.: Response surface methodology and the design of clinical trials for the evaluation of cancer chemotherapy. Cancer Treat. Rep. 69: 1049–1051, 1985.
86. Carter, W. H., Jr., Chinchilli, V. M., Campbell, E. D., and Wampler, G. L.: Confidence interval about the response at the stationary point of a response surface, with an application to preclinical cancer therapy. Biometrics 40: 1125–1130, 1984.
87. Carter, W. H., Jr., Gennings, C., Staniswalis, J. G., Campbell, E. D., and White, K. L., Jr.: A statistical approach to the construction and analysis of isobolograms. J. Am. Coll. Toxicol. 7: 963–973, 1988.
88. Carter, W. H., Jr., Jones, D. E., and Carchman, A. A.: Application of response surface methods for evaluating the interactions of soman, atropine, and pralidioxime chloride. Fundam. Appl. Toxicol. 5: S232–S241, 1985.
89. Carter, W. H., Jr., Stablein, D. M., and Wampler, G. L.: An improved method for analyzing survival data from combination chemotherapy experiments. Cancer Res. 39: 3446–3453, 1979.
90. Carter, W. H., Jr., and Wampler G. L.: Review of the application of response surface methodology in the combination therapy of cancer. Cancer Treat. Rep. 70: 133–140, 1986.
91. Carter, W. H., Jr., Wampler, G. L., Crews, S. L., and Howells, R.: On determining the levels of treatment to optimize the probability of a favorable response. Cancer Treat. Rep. 61: 849–853, 1977.
92. Carter, W. H., Jr., Wampler, G. L., and Stablein, D. M.: Response Analysis of Survival Data in Cancer Chemotherapy. Marcel Dekker Inc., New York, 1983.
93. Carter, W. H., Jr., Wampler, G., Stablein, D. M., and Campbell, E. D.: Drug activity and therapeutic synergism in cancer treatment. Cancer Res. 42: 2963–2971, 1982.
94. Cartwright, A., and Smith, C.: Elderly People, Their Medicines and Their Doctors. Routledge, London, 1988.
95. Cassatella, M. A., Hartman, L., Perussia, B., and Trinchieri, G.: Tumor necrosis factor and immune interferon synergistically induce cytochrome b_{-245} heavy chain gene expression and nicotinamide-adenine dinucleotide phosphate hydrogenase oxidase in human leukemic myeloid cells. J. Clin. Invest. 83: 1570–1579, 1989.
96. Cavalieri, E., Munhall, A., Rogan, E., Salmasi, S., and Patil, K.: Syncarcinogenic effect of the environmental pollutants cyclo-penteno[cd] pyrene and benzo[a]pyrene in mouse skin. Carcinogenesis 4: 393–397, 1983.
97. Chabbert, Y.: Action des associations d'antibiotiques sur les germes aérobes. Ann. Inst. Pasteur 84: 545–561, 1953.
98. Chang, B. K., Gutman, R., and Chou, T-C.: Schedule-dependent interaction of α-difluoromethylornithine and *cis*-diamminedichoroplatinum (II) against human and hamster pancreatic cell lines. Cancer Res. 47: 2247–2250, 1987.
99. Chang, T-T., Gulati, S., Chou, T-C., Colvin, M., and Clarkson, B.: Comparative cytotoxicity of various drug combinations for human leukemic cells and normal hematopoietic precursors. Cancer Res. 47: 119–122, 1987.
100. Chang, T-T., Gulati, S. C., Chou, T-C., Vega, R., Gandola, L., Ezzat Ibrahim, S. M., Yopp, J., Colvin, M., and Clarkson, B. D.: Synergistic effect of 4-hydroperoxycyclophosphamide and etoposide on a human promyelocyte leukemia cell line (HL-60) demonstrated by computer analysis. Cancer Res. 45: 2434–2439, 1985.
101. Changeux, J-P., and Podleski, T. R.: On the excitability and cooperativity of the electroplax membrane. Proc. Nat. Acad. Sci. 59: 944–950, 1968.
102. Chapekar, M. S., Hartman, K. D., Knode, M. C., and Glazer, R. I.: Synergistic effect of retinoic acid and calcium ionophore A23187 on differentiation, c-*myc* expression, and membrane tyrosine kinase activity in human promyelocytic leukemia cell line HL-60. Mol. Pharmacol. 31: 140–145, 1987.
103. Chapin, R. E., Phelps, J. L., Schwetz, B. A., and Yang, R. S. H.: Toxicology studies of a chemical mixture of 25 groundwater contaminants: (III) Male reproduction study in $B6C3F_1$ mice. Fund. Appl. Toxicol., in press.
104. Chawira, A. N., Warhurst, D. C., Robinson, B. L., and Peters, W.: The effect of combinations of ginghaosu (artemisinin) with standard antimalarial drugs in the suppressive treatment of malaria in mice. Trans. Roy. Soc. Trop. Med. Hyg. 81: 554–558, 1987.
105. Chen, G., and Ensor, C. R.: The combined anticonvulsant activity and

UNITED THERAPEUTICS CORP., EX1034, page 40
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

toxicity of dilantin and n-methyl-5-phenylsuccinamide. J. Lab. Clin. Med. **41**: 78–83, 1953.

106. CHEN, J. J., HASS, B. S., AND HEFLICH, R. H.: A response-additive model for assessing the joint action of mixtures. *In* ASA/EPA Conferences on Interpretation of Environmental Data. The Current Assessment of Combined Toxicant Effects. May 5–6, 1986. USEPA, Washington, D.C., pp.78–81, 1986.
107. CHOU, T-C.: Derivation and properties of Michaelis-Menten type and Hill type equations for reference ligands. J. Theor. Biol. **59**: 253–276, 1976.
108. CHOU, T-C.: On the determination of availability of ligand binding sites in steady-state systems. J. Theor. Biol. **65**: 345–356, 1977.
109. CHOU, T-C.: Comparison of dose-effect relationships of carcinogens following low-dose chronic exposure and high-dose single injection: an analysis by the median effect principle. Carcinogenesis **1**: 203–213, 1980.
110. CHOU, T-C., AND TALALAY, P.: A simple generalized equation for the analysis of mutliple inhibitions of Michaelis-Menten kinetic systems. J. Biol. Chem. **252**: 6438–6442, 1977.
111. CHOU, T-C., AND TALALAY, P.: Generalized equations for the analysis of Michaelis-Menten and higher-order kinetic systems with two or more mutually exclusive and non-exclusive inhibitors. Eur. J. Biochem. **115**: 207–216, 1981.
112. CHOU, T-C., AND TALALAY, P.: Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzyme Reg. **22**: 27–55, 1984.
113. CHOU, T-C., AND TALALAY, P.: Applications of the Median-Effect Principle for the assessment of low-dose risk of carcinogens and for the quantitation of synergism and antagonism of chemotherapeutic agents. *In* New Avenues in Developmental Cancer Chemotherapy, ed. by K. R. Harrap, and T. A. Connors, pp. 37–64, Academic Press, New York, 1987.
114. CHOW, A. W., WONG, J., AND BARTLETT, K. H.: Synergistic interactions of ciprofloxacin and extended-spectrum β-lactams or aminoglycosides against multiply drug-resistant *Pseudomonas maltophilia*. Antibiot. Agents Chemother. **32**: 782–784, 1988.
115. CHRISTENSEN, E. A., AND CHEN, C-Y.: A general noninteractive multiple toxicity model including probit, logit, and Weibull transformations. Biometrics **41**: 711–725, 1985.
116. CLARK, A. J.: The reaction between acetylcholine and muscle cells. J. Physiol. **16**: 530–547, 1926.
117. CLARK, A. J.: The antagonism of acetylcholine by atropine. J. Physiol. **16**: 548–556, 1926.
118. CLEJAN, L., AND CEDERBAUM, A. I.: Synergistic interactions between NADPH-cytochrome P-450 reductase, paraqat, and iron in the generation of active oxygen radicals. Biochem. Pharmacol. **38**: 1779–1786, 1989.
119. CLUFF, L. E., THORNTON, G. F., AND SEIDL, L. G.: Studies on the epidemiology of adverse drug reactions. I. Methods of surveillance. J. Am. Med. Assoc. **188**: 976–983, 1964.
120. COHEN, J. D., AND ROBINS, H. I.: Hyperthermic enhancement of cis-diammine-1,1-cyclobutane dicarboxylate platinum (II) cytotoxicity in human leukemic cells *in vitro*. Cancer Res. **47**: 4335–4337, 1987.
121. COHEN, J. D., ROBINS, H. I., MULCAHY, R. T., GIPP, J. J., AND BOUCK, N.: Interactions between hyperthermia and irradiation in two human lymphoblastic leukemia cell lines *in vitro*. Cancer Res.: **48**: 3576–3580, 1988.
122. COLLEN, D.: Synergism of thrombolytic agents: investigational procedures and clinical potential. Circulation **77**: 731–735, 1988.
123. COLLEN, D., STUMP, D. C., AND VAN DER WERF, F.: Coronary thrombolysis in patients with acute myocardial infarction by intravenous infusion of synergic thrombolytic agents. Am. Heart J. **112**: 1083–1084, 1986.
124. COLLEN, D., AND VAN DER WERF, F.: Coronary arterial thrombolysis with low-dose synergistic combinations of recombinant tissue-type plasminogen activator (rt-PA) and recombinant single-chain urokinase-type plasminogen activiator (rscu-PA) for acute myocardial infarction. Am. J. Cardiol. **60**: 431–434, 1987.
125. COMMITTEE ON METHODS FOR THE IN VIVO TOXICITY TESTING OF COMPLEX MIXTURES. NATIONAL RESEARCH COUNCIL.: Complex Mixtures. Methods for In Vivo Toxicity Testing. National Academy Press, Washington, D.C., 1988.
126. CONNELL, E. V., CERRUTI, R. L., AND TROWN, P. W.: Synergistic activity of combinations of recombinant human alpha interferon and acyclovir, administered concomitantly and in sequence, against a lethal herpes simplex virus type 1 infection in mice. Antibiot. Agents Chemother. **28**: 1–4, 1985.
127. CRANE, L. R., AND MILNE, D. A.: Comparative activities of combinations of acyclovir, vidarabine or its 5′-monophosphate, and cloned human interferons against herpes simplex virus type 2 in human and mouse fibroblast cultures. Antiviral Res. **5**: 325–333, 1985.
128. CRATHORN, A. R., AND ROBERTS, J. J.: Reactions of cultured mammalian cells of varying radiosensitivity with the radiomimetic alkylating agent, mustard gas. Prog. Biochem. Pharmacol. **1**: 320–326, 1965.
129. CROWLE, A. J., SALFINGER, M., AND MAY, M. H.: 1,25(OH)-vitamin D3 synergizes with pyrazinamide to kill tubercle bacilli in cultured human macrophages. Am. Rev. Respir. Dis. **139**: 549–552, 1989.
130. CROWTHER, J. A.: Some considerations relative to the action of X-rays on tissue cells. Proc. Roy. Soc. B. **96**: 207–211, 1924.
131. CRUMP, K. S., HOEL, D. G., LANGLEY, C. H., AND PETO, R.: Fundamental carcinogenic processes and their implications for low dose risk assessment. Cancer Res. **36**: 2973–2979, 1976.
132. CURRY, S. H.: Homergic interactions, isobols and drug concentrations in blood. *In* Drug Interactions, ed. by D. G. Grahame-Smith, pp. 87–99, Macmillan Press, London, 1977.
133. CZARNIECKI, C. W., FENNIE, C. W., POWERS, D. B., AND ESTELL, D. A.: Synergistic antiviral and antiproliferative activities of *Escherichia-coli*-derived human alpha, beta, and gamma interferons. J. Virol. **49**: 490–496, 1984.
134. DAHL, R., PEDERSEN, B., AND HÄGGLÖF, B.: Nocturnal asthma: effect of treatment with oral sustained-release terbutaline, inhaled budesonide, and the two in combination. J. Allergy Clin. Immunol. **83**: 811–815, 1989.
135. DALE, M. M., AND PENFIELD, A.: Synergism between phorbol ester and A23187 in superoxide production by neutrophils. FEBS Lett. **175**: 170–172, 1984.
136. DE CHAUFFOY DE COURCELLES, D., ROEVENS, P., VAN BELLE, H., AND DE CLERK, F.: The synergistic effect of serotonin and epinephrine on the human platelet at the level of signal transduction. FEBS Lett. **219**: 283–288, 1987.
137. DEEN, D. F., AND WILLIAMS, M. E.: Isobologram analysis of X-ray-BCNU interactions *in vitro*. Radiat. Res. **79**: 483–491, 1979.
138. DE FLORA, S.: A "spiral test" applied to bacterial mutagenesis assays. Mutat. Res. **82**: 213–227, 1981.
139. DE MAEYER, E., AND DE MAEYER-GUIGNARD, J.: Interferons and other regulatory cytokines. John Wiley, New York, 1988.
140. DENZ, H., LECHLEITNER, M., MARTH, C. H., DAXENBICHLER, G., GASTL, G., AND BRAUNSTEINER, H.: Effect of human recombinant alpha-2-and gamma-interferon on the growth of human cell lines from solid tumors and hematologic malignancies. J. Interferon Res. **5**: 147–157, 1985.
141. D'ESOPO, N. D.: Clinical trials in pulmonary tuberculosis. Am. Rev. Respir. Dis. Suppl. 85, **125**: 85–93, 1982.
142. DESPOIS, R., AND PINNERT, S.: Potentiation de la pénicilline par différents antibiotiques *in vitro*. Comptes Rend. Soc. Biol. **144**: 23–25, 1950.
143. DE VITA, V. T., JR., HUBBARD, S. M., YOUNG, R. C., AND LONGO, D. L.: The role of chemotherapy in diffuse aggressive lymphomas. Semin. Hematol. Suppl. 2, **25**: 2–10, 1988.
144. DE WAARD, M.A.: Synergism and antagonism in fungicides. *In* Modern Selective Fungicides—Properties, Applications, Mechanisms of Action, ed. by H. Lyr, pp. 355–365, Longmans Group UK Ltd., London, 1987.
145. DEWEY, W. C., STONE, L. E., MILLER, H. H., AND GIBLAK, R. E.: Radiosensitization with 5-bromodeoxyuridine of Chinese hamster cells X-irradiated during different phases of the cell cycle. Radiat. Res. **47**: 672–688, 1971.
146. DEXTER, T. M.: Haemopoietic growth factors. Brit. Med. Bull. **45**: 337–349, 1989.
147. DOLL, R.: Strategy for detection of cancer hazards to man. Nature (Lond.) **265**: 589–596, 1977.
147a. DONAHUE, R. E., SEEHRA, J., METZGER, M., LEFEBRE, D., ROCK, B., CARBONE, S., NATHAN, D. G., GARNICK, M., SEHGAL, P. K., LASTON, D., LAVALLIE, E., MCCOY, J., SCHENDEL, P. F., NORTON, C., TURNER, K., YANG, Y-C., AND CLARK, S. C.: Human IL-3 and GM-CSF act synergistically in stimulating hematopoiesis in primates. Science (Wash., DC) **241**: 1820–1823, 1988.
148. DORRINGTON, J., CHUMA, A. V., AND BENDELL, J. J.: Transforming growth factor and follicle-stimulating hormone promote rat granulosa cell proliferation. Endocrinology **123**: 353–359, 1988.
149. DRASKÓCZY, P. A., AND TRENDELENBURG, U.: The uptake of l-and d-norepinephrine by the isolated perfused rabbit heart in relation to the stereospecificity of the sensitizing action of cocaine. J. Pharmacol. Exp. Ther. **159**: 66–73, 1968.
150. DREUX, C., IMHOFF, V., AND ROSSIGNOL, B.: [3H] protein secretion in rat parotid gland: substance P-β-adrenergic synergism. Am. J. Physiol. **253**: C774-C782, 1987.
151. DREWINKO, B., LOO, T. B., BROWN, B., GOTTLIEB, J. A., AND FREIREICH, E. J.: Combination chemotherapy *in vitro* with adriamycin. Observations of additive, antagonistic, and synergistic effects when used in two-drug combinations on cultured human lymphoma cells. Cancer Biochem. Biophys. **1**: 187–195, 1976.
152. DRUILHE, P., BRANDICOURT, O., CHONGSUPHAJAISIDDHI, T., AND BERTHE, J.: Activity of a combination of three cinchona bark alkaloids against *Plasmodium falciparum* in vitro. Antibiot. Agents Chemother. **32**: 250–254, 1988.
153. DURAND, R. E., AND CHAPLIN, D. J.: Chemosensitization by misonidazole in CCNU-treated spheroids and tumours. Br. J. Cancer **56**: 103–109, 1987.
154. DURAND, R. E., AND GOLDIE, J. H.: Interaction of etoposide and cisplatin in an in vitro tumor model. Cancer Treat. Rep. **71**: 673–679, 1987.
155. DURAND, R. E., AND OLIVE, P. L.: Enhancement of toxicity from N-(2-chloroethyl)-N′-cyclohexyl-N-nitrosourea in V79 spheroids by a nitrofuran. Cancer Res. **47**: 5303–5309, 1987.
156. DYSON, A. J., AND CAMPBELL, I. A.: Interaction between choline theophylline and salmefamol in patients and reversible airways obstruction. Br. J. Clin. Pharmacol. **4**: 677–682, 1977.
157. EHRENBERG, L., MOUSTACCHI, E., OSTERMAN-GOLKAR, S., AND EKMAN,

G.: Dosimetry of genotoxic agents and dose-response relationships of their effects. Mutat. Res. 123: 121–182, 1983.

158. ELIAS, J. A.: Tumor necrosis factor interacts with interleukin-1 and interferons to inhibit fibroblast proliferation via fibroblast prostaglandin-dependent and -independent mechanisms. Am. Rev. Respir. Dis. 138: 652–658, 1988.

159. ELIAS, J. A., KROL, R. C., FREUNDLICH, S., AND SAMPSON, P. M.: Regulation of human lung fibroblast glycosaminoglycan production by recombinant interferons, tumor necrosis factor, and lymphotoxin. J. Clin. Invest. 81: 325–333, 1988.

160. ELION, G. B., SINGER, S., AND HITCHINGS, G. H.: Antagonists of nucleic acid derivatives. VIII. Synergism in combinations of biochemically related antimetabolites. J. Biol. Chem. 208: 477–488, 1954.

161. ELKIND, N. M., UTSUMI, H., AND BEN-HUR, E.: Are single or multiple mechanisms involved in radiation-induced mammalian cell killing? Br. J. Cancer Supp. VIII 55: 24–31, 1987.

162. ELKIND, M. M., AND WHITMORE, G. F.: The Radiobiology of Cultured Mammalian Cells. Gordon and Breach, New York, 1967.

163. ELLARD, G. A.: Chemotherapy of leprosy. Br. Med. Bull. 44: 775–790, 1988.

164. ELLIOTT, G. R., TAK, C., AND BONTA, I. L.: Prostaglandin E_2 and leukotriene C_4 modulate interleukin-1 inhibition of WEHI-3B tumor cell growth *in vitro*. Agents and Action 26: 165–166, 1989.

164a. ELLIS, M. N., LOBE, D. C., AND SPECTOR, T.: Synergistic therapy by acyclovir and A111OU for mice orofacially infected with herpes simplex viruses. Antibiot. Agents Chemother. 33: 1691–1696, 1989.

165. ELOFSON, S-A., GAMBERALE, F., HINDMARSH, T., IREGREN, A., ISAKSSON, A., JOHNSSON, I., KNAVE., B., LYDAHL, E., MINDUS, P., PERSSON, H. E., PHILPSON, B., STEBY, M., STRUVE, G., SÖDERMAN, E., WENNBERG, A., AND WIDÉN, L.: Exposure to organic solvents. A cross-sectional epidemiologic investigation on occupationally exposed car and industrial spray painters with special reference to the nervous system. Scand. J. Work Environ. Health 3: 239–273, 1980.

166. ENNIST, D. L., ELKINS, K. L., CHENG, S. C., AND HOWARD, M.: Activity of a partially purified human BCFG on murine assays for B cell stimulatory factors. II. Synergy between two distinct BCGF II-like factors in promoting B cell differentiation. J. Immunol. 139: 1525–1531, 1987.

167. ERIKSSON, B. F. H., AND SCHINAZI, R. F.: Combinations of 3′-azido-3′ deoxythymidine (Zidovudine) and phosphonoformate (Foscarnet) against against human immunodeficiency virus type 1 and cytomegalovirus replication in vitro. Antibiot. Agents Chemother. 33: 663–669, 1989.

168. ESPARZA, I., MÄNNEL, D., RUPPEL, A., FALK, W., AND KRAMMER, P.H.: Interferon and lymphotoxin or tumor necrosis factor act synergistically to induce macrophage killing of tumor cells and schistosomata of *Schistosoma mansoni*. J. Exp. Med. 166: 589–594, 1987.

169. FALK, L. A., AND VOGEL, S. N.: Granulocyte-macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (CSF-1) synergize to stimulate progenitor cells with high proliferative potential. J. Leucocyte Biol. 44: 455–464, 1988.

170. FEARN, H. J., AND HODGES, J. R.: Synergic effects of amylobarbitone sodium and ethanol. J. Pharm. Pharmacol. 5: 1041–1044, 1953.

171. FEDELLI, L., MENEGHINI, L., SANGIOVANNI, M., SCROLLINI, F., AND GORI, E.: Quantitative evaluation of joint drug action. *In* Toxicological Problems of Drug Combinations, ed. by S.N. De C. Baker, pp. 231–245, Excerpta Medica, Amsterdam, 1971.

172. FELDMAN, W. E.: Effect of ampicillin and chloramphenicol against *Haemophilus influenzae*. Pediatrics 61: 406–409, 1978.

173. FERNANDEZ-BOTRAN, R., SANDERS, V. M., MOSMANN, T. R., AND VITETTA, E. S.: Lymphokine-mediated regulation of the proliferative response of clones of T helper 1 and T helper 2 cells. J. Exp. Med. 168: 543–558, 1988.

174. FERNANDEZ-BOTRAN, R., SANDERS, V. M., AND VITETTA, E. S.: Interactions between receptors for interleukin 2 and interleukin 4 on lines of helper T cells (HT-2) and B lymphoma cells (BCL_1). J. Exp. Med. 169: 379–391, 1989.

175. FINNEY, D. J.: The analysis of toxicity tests on mixtures of poisons. Ann. Appl. Biol 29: 82–94, 1942.

176. FINNEY, D. J.: Probit Analysis (second edition), Cambridge University Press, Cambridge, 1952.

177. FINNEY, D. J.: Probit Analysis (third edition), Cambridge University Press, Cambridge, 1971.

178. FLEISCHMANN, W. R., JR., FLEISCHMANN, C. M., AND GINDHART, T. D.: Effect of hyperthermia on combination interferon treatment: enhancement of the antiproliferative activity against murine B-16 melanoma. Cancer Res. 46: 1722–1726, 1986.

179. FLORES, B. A., BISTRIAN, B. R., POMPOSELLI, J. J., DINARELLO. C. A., BLACKBURN, G. L., AND ISTFAN, W.: Infusion of tumor necrosis factor/cachectin promotes muscle catabolism in the rat. A synergistic effect with interleukin 1. J. Clin. Invest. 83: 1614–1622, 1989.

180. FOX, M., AND NIAS, A. H. W.: A modification of the sensitivity of mammalian cells surviving treatment with methyl methanesulphonate. Eur. J. Cancer 4: 325–335, 1968.

181. FOX, W., AND MITCHISON, D.A.: Short-course chemotherapy for pulmonary tuberculosis. Am. Rev. Resp. Dis. 111: 325–353, 1975.

182. FRASER, T. R.: An experimental research on the antagonism between the actions of physostigma and atropia. Proc. Roy. Soc. Edin. 7: 506–511, 1870–1871.

183. FRASER, T. R.: The antagonism between the actions of active substances. Br. Med. J. 2: 485–487, 1872.

184. FREI, E., III, AND GOTTLIEB J. A.: Combination chemotherapy: clinical considerations. *In* Antineoplastic and Immunosuppressive Agents. Part 1. Handbook of Experimental Pharmacology, ed. by A. C. Sartorelli and D. C. Johns, pp. 449–467, Springer-Verlag, Berlin, 1974.

185. FREI, W.: Versuche über Kombination von Desinfektionsmitteln. Z. Hyg. 75: 433–496, 1913.

186. FRY, M., AND WILLIAMS, R. B.: Effects of decoquinate and clopidol on electron transport in mitochondria of *Eimeria tenella* (Apicomplexa: Coccidia). Biochem. Pharmacol. 33: 229–240, 1984.

187. FU, K. P., AND NEU, H. C.: In vitro synergistic effect of netilmicin, a new aminoglycoside antibiotic. Antibiot. Agents Chemother. 10: 511–518, 1976.

188. FULLER, R. W., DIXON, C. M. S., DOLLERY, C. T., AND BARNES, P. J.: Prostaglandin D_2 potentiates airway responsiveness to histamine and methacholine. Am. Rev. Respir. Dis. 133: 252–254, 1986.

189. GADDUM, J. H.: Pharmacology, Oxford University Press, London, 1940.

190. GALIVAN, J., NIMEC, Z., RHEE, M., BOSCHELLI, D., ORONSKY, A. L., AND KERWAR, S. S.: Antifolate drug interactions: enhancement of growth inhibition due to the antipurine 5,10-dideazothetrahydrofolic acid by the lipophilic dihydrofolate reductase inhibitors metoprine and trimetrexate. Cancer Res. 48: 2421–2425, 1988.

191. GARBARG, M., AND SCHWARTZ, J-C.: Synergism bewteen histamine H_1-and H_2- receptors in the cAMP response in guinea pig brain slices: effects of phorbol esters and calcium. Mol. Pharmacol. 33: 38–43, 1988.

192. GARDNER, M. J., AND MUNFORD, A. G.: The combined effect of two factors on disease in a case-control study. Appl. Stat. 29: 276–281, 1980.

193. GARRETT, E. R.: Classification and evaluation of combined antibiotic activity. Antibiot. Chemother. 8: 8–20, 1958.

194. GARRETT, E. R.: The use of microbial kinetics in the quantification of antibiotic action. Arzneimittelforschung 16: 1364–1369, 1966.

195. GARRETT, E. R., WRIGHT, O. K., MILLER, G. H., AND SMITH, K. L.: Quantification and prediction of the biological activities of chloramphenicol analogs by microbial kinetics. J. Med. Chem. 9: 203–208, 1966.

196. GATELY, M. K., WILSON, D. E., AND WONG, H. L.: Synergism between recombinant interleukin 2 (rIL2) and IL2-depleted lymphokine-containing supernates in facilitating allogeneic human cytolytic T lymphocyte responses in vitro. J. Immunol. 136: 1274–1282, 1986.

197. GAZZANIGA, P. P., DIMARCO G., LA MANAISA P., ODDI, A., PAPPALARDO, G., PULCINELLI., F. M., AND REGGIO, D.: In vitro enhancement of human platelet aggregation by somatostatin. Experientia 44: 892–894, 1988.

198. GENNINGS, C., CARCHMAN, R. A., CARTER, W. H., JR., CAMPBELL, E. D., BOYLE, R. M., STANISWALIS, J. G., JONES, D. E., REID, F. M., AND KOPLOVITZ, I.: Assessing physostigmine efficacy by response surface modelling: a comparison to pyridostigmine efficacy. J. Am. Coll. Toxicol. 7: 1013–1030, 1988.

199. GERMOLEC, D. R., YANG., R. S. H., ACKERMANN, M. F., ROSENTHAL, G. J., BOORMAN, G. A., BLAIR, P., AND LUSTER, M. I.: Toxicology studies of a chemical mixture of 25 groundwater contaminants: (II) Immunosuppression in $B6C3F_1$ mice. Fund. Appl. Toxicol. In press.

199a. GESEK, F. A., CRAGOE, E. J. JR., AND STRANDHOY, J. W.: Synergistic α-1 and α-2 adrenergic stimulation of rat proximal nephron Na^+/H^+ exchange. J. Pharm. Exp. Ther. 249: 694–700, 1989.

200. GESSNER, P. K.: The isobolographic method applied to drug interactions. *In* Drug Interactions, ed. by P. L. Morselli, S. Garattini, and S. N. Cohen, pp. 349–362, Raven Press, New York, 1974.

201. GESSNER, P. K.: A straightforward method for the study of drug interactions: an isobolographic analysis primer. J. Am. Coll. Toxicol. 7: 987–1012, 1988.

202. GESSNER, P. K., AND CABANA, B. E.: A study of the interactions of the hypnotic effects and of the toxic effects of chloral hydrate and ethanol. J. Pharmacol. Exp. Ther. 174: 247–259, 1970.

203. GESSNER, P. K., AND GESSNER, T.: The interaction between barbital and testosterone relative to their hypnotic effect. Arch. Int. Pharmacodyn. 201: 52–58, 1973.

204. GESSNER, P. K., AND SHAKARJIAN, M. P.: Interactions of paraldehyde with ethanol and chloral hydrate. J. Pharmacol. Exp. Ther. 235: 32–36, 1985.

205. GESSNER, P. K. AND SOBLE, A. G.: A study of the transylcypromine-mepiridine interaction: effects of p-chlorphenylalanine and l-5-hydroxytryptophan. J. Pharmacol. Exp. Ther. 186: 276–287, 1973.

206. GILCHRIST, W. J., LEE, Y. C., TAM, H. C., MACDONALD, J. B., AND WILLIAMS, B. O.: Prospective study of drug reporting by general practitioners for an elderly population referred to a geriatric service. Br. Med. J. 294: 289–290, 1987.

207. GILLIES, G. E., LINTON, E. A., AND LOWRY, P. J.: Corticotropin releasing activity of the new CRF is potentiated several times by vasopressin. Nature 299: 355–357, 1982.

208. GLOBUS, R. K., PATTERSON-BUCKENDAHL, P., AND GOSPODAROWICZ, D.: Regulation of bovine cell proliferation by fibroblast growth factor and transforming growth factor. Endocrinology 123: 98–105, 1988.

209. GOLDIN, A., AND MANTEL, N.: The employment of combinations of drugs

in the chemotherapy of neoplasia: a review. Cancer Res. 17: 635–654, 1957.

210. GOLDIN, A., VENDITTI, J. M., AND MANTEL, N. Combination chemotherapy: basic considerations. *In* Antineoplastic and Immunosuppressive Agents, Part 1. Handbook of Experimental Pharmacology, ed. by A. C. Sartorelli and D. C. Johns, pp. 411–448, Springer-Verlag, Berlin, 1974.

211. GOLDSTEIN, L.: Kinetic behavior of immobilized enzyme systems. In Methods in Enzymology, ed. by K. Mosbach, Vol. XLIV, Immobilized Enzymes, pp. 397–443, Academic Press, New York, 1976.

212. GOODMAN, M. G.: Interaction between cytokines and 8-mercaptoguanosine in humoral immunity: synergy with interferon. J. Immunol. 139: 142–146, 1987.

213. GRANT, J. A., AND SCRUTTON, M. C.: Positive interaction between agonists in the aggregation response of human blood platelets: interaction between ADP, adrenaline and vasopressin. Br. J. Haematol. 44: 109–125, 1980.

214. CRECO, F. A., JOHNSON, D. H., HAINSWORTH, J. D., AND WOLFF, S. N.: Chemotherapy of small-cell lung cancer. Semin. Oncol. Supp. 6 12: 31-37, 1985.

215. CRINDEY, G. B., MORAN, R. G., AND WERKHEISER, W. C.: Approaches to the rational combination of antimetabolites for cancer chemotherapy. *In* Drug Design, Vol. 5, ed. by E. J. Ariëns, pp. 169–249, Academic Press, New York, 1975.

216. GRINDEY, G. B., AND NICHOL, C. A.: Interaction of drugs inhibiting different steps in the synthesis of DNA. Cancer Res. 32: 527–531, 1972.

217. GROLL-KNAPP, E., HAIDER, M., KIENZL, K., HANDLER, A., AND TRIMMEL, M.: Changes in discrimination learning and brain activity (ERP's) due to combined exposure to NO and CO in rats. Toxicology 49: 441–447, 1988.

218. GRUNNET, N., VIND C., AND DICH, J.: Synergistic effect of insulin and glucocorticoid on albumin secretion in cultured hepatocytes. Acta Physiol. Scand. 133: 593–594, 1988.

219. GUESS, H. A., AND CRUMP, K.: Best-estimate low-dose extrapolation of carcinogenicity data. Environ. Health Perspect. 22: 149–152, 1978.

220. GUESS, H., CRUMP, K., AND PETO, R.: Uncertainty estimates for low-dose-rate extrapolations of animal carcinogenicity data. Cancer Res. 37: 3475–3483, 1977.

221. GUIMEZANES, A., BUFERNE, M., PONT, S., PEIRRES, M., AND SCHMITT-VERHULST, A. M.: Interactions between the Thy-I and T-cell antigen receptor pathways in the activation of cytotoxic T cells: evidence for synergistic effects, loss variants, and anti-CD8 antibody-mediated inhibition. Cell. Immunol. 113: 435–446, 1988.

222. HAHON, N., AND BOOTH, J. A.: Coinhibition of viral interferon induction by benzo[a]pyrene and chrysotile asbestos. Environ. Res. 19: 103–109, 1986.

223. HALL, M. J., MIDDLETON, R. F., AND WESTMACOTT, D.: The fractional inhibitory concentration (FIC) index as a measure of synergy. J. Antimicrob. Chemother. 11: 427–433, 1983.

224. HALLANDER, H. O., DORNBUSCH, K., GEZELIUS, L., JACOBSON, K., AND KARLSSON, I.: Synergism between aminoglycosides and cephalosporins with antipseudomonal activity: interaction index and killing curve method. Antimicrob. Agents Chemother. 22: 743–752, 1982.

225. HAMILTON-MILLER, J. M. T.: Rationalization of terminology and methodology in the study of antibiotic interactions. J. Antimicrob. Chemother. 15: 655–658, 1985.

226. HAMMER, S. M., AND GILLIS, J. M.: Synergistic activity of granulocyte-macrophage colony-stimulating factor and 3-azido-3'-deoxythymidine against human immunodeficiency virus in vitro. Antimicrob. Agents Chemother. 31: 1046–1050, 1987.

227. HAN, A., AND ELKIND, M. M.: Ultraviolet light and X-ray damage interaction in Chinese hamster cells. Radiat. Res. 74: 88–100, 1978.

228. HANLEY, C. A., ELIAS, R. M., MOVAT, H. Z., AND JOHNSTON, M. G.: Suppression of fluid pumping in isolated bovine lymphatics by interleukin-1: Interaction with prostaglandin E_2. Microvasc. Res. 37: 218–229, 1989.

229. HÄNNINEN, H., ESKELINEN, L., HUSMAN, K. AND NURMINEN, M.: Behavioral effects of long-term exposure to a mixture of organic solvents. Scand. J. Work Environ. Health 4: 240–255, 1976.

230. HARRISON, P. T. C., AND HEATH, J. C.: Apparent synergy between chrysotile asbestos and N-nitrosoheptanemethyleneamine in the induction of pulmonary tumours in rats. Carcinogenesis 9: 2165–2171, 1988.

231. HARSTHORN, K. L., SANDSTROM, E. G., NEUMEYER, D., PARADIS, T. J., CHOU, T-C., SCHOOLEY, R. T., AND HIRSCH, M. S.: Synergistic inhibition of human T-cell lymphotropic virus type III replication in vitro by phosphonoformate and recombinant alpha-A interferon. Antibiot. Agents Chemother. 30: 189–191, 1986.

232. HARTSHORN, K. L., VOGT, M. W., CHOU, T-C., BLUMBERG, R. S., BYINGTON, R., SCHOOLEY, R. T., AND HIRSCH, M. S.: Synergistic inhibition of human immunodeficiency virus in vitro by azidothymidine and recombinant alpha A interferon. Antibiot. Agents Chemother. 31: 168–172, 1987.

233. HARTZELL, H. C., AND FISCHMEISTER, R.: Effect of forskolin and acetylcholine on calcium current in single isolated cardiac myocytes. Mol. Pharmacol. 32: 639–645, 1987.

234. HARVEY, M. J., AND KLAASSEN, C. D.: Interaction of metals and carbon tetrachloride on lipid peroxidation and hepatotoxicity. Toxicol. Appl. Pharmacol. 71: 316–322, 1983.

235. HARVEY, R. J.: Interaction of two inhibitors which act on different enzymes of a metabolic pathway. J. Theor. Biol. 74: 411–437, 1978.

236. HASLAM, R. J., WILLIAMS, K. A., AND DAVIDSON, M. M. L.: Receptor-effector coupling in platelets: roles of guanine nucleotides. *In* Mechanisms of Stimulus-Response Coupling in Platelets, ed. by J. Westwick, M. F.Scully, D. E. MacIntyre, and V. V. Kakkar, pp. 160–173, Plenum Press, New York, 1985.

237. HASS, B. S., CHEN, J. J., CHOU, M. W., FU, P. P., AND HEFLICH, R. H.: Binary mixtures containing isomers of nitrobenzo[a]pyrene induce greater-than-additive mutational responses in *Salmonella typhimurium*. Mutat. Res. 190: 247–252, 1987.

238. HAYASHI, Y., SUMIKAWA, K., TASHIRO, C., AND YOSHIYA, I.: Synergistic interaction of α-1 and β-adrenoceptor agonists on induction arrhythmias during halothane anesthesia in dogs. Anesthesiology 68: 902–907, 1988.

239. HEIFETS, L. B.: Synergistic effect of rifampicin, streptomycin, ethionamide, and ethambutol on *Mycobacterium intracellulare*. Am. Rev. Respir. Dis. 125: 43–48, 1982.

240. HEIFETS, L. B., ISEMAN, M. D., AND LINDHOLM-LEVY, P. J.: Combinations of rifamycin or rifabutine against *Mycobacterium avium* complex. Am. Rev. Respir. Dis. 137: 711–715, 1988.

241. HELLEWELL, P. G., AND WILLIAMS, T. J.: Interactions between eicosanoids and other mediators of inflammation. *In* Eicosanoids in Inflammatory Conditions of the Lung, Skin and Joints, ed. by M. Church and C. Robinson, pp. 43–66, MTP Press Ltd., Lancaster, 1988.

242. HENDERSON, D. W., AND GORER, P. A.: The treatment of certain experimental anaerobic infections with sulphapyridine and with immune sera and the problem of synergic action. J. Hyg. 40: 345–364, 1940.

243. HENDERSON, E. S., AND SAMAHA, R. J.: Evidence that drugs in multiple combinations have materially advanced the treatment of human malignancies. Cancer Res. 29: 2272–2280, 1969.

244. HENKEL, W., AND MLETZKO, G.: Input-Untersuchung der kombinierten Lärm—und Schwingungsbelastung von Ratten. Z. ges. Hyg. 21: 8–10, 1975.

245. HENLE, A.: Ueber Creolin und seine wirksamen Bestandtheile. Arch. f. Hyg. 9: 188–222, 1889.

246. HERNANDEZ E., AND ROSENSHEIN, N. B.: Interaction between bisantrene and radiation. Int. J. Radiat. Oncol. Biol. Phys. 11: 1395–1399, 1985.

247. HEWLETT, P. S.: Measure of the potencies of drug mixtures. Biometrics 25: 477–487, 1969.

248. HEWLETT, P. S.: The toxicity to *Tribolium castaneum* (Herbst) (Coleoptera, Tenebrionidae) of mixtures of pyrethrins and piperonyl butoxide: fitting a mathematical model. J. Stored Prod. Res. 5: 1–9, 1969.

249. HEWLETT, P. S., AND PLACKETT, R. L.: Similar joint action of insecticides. Nature 169: 198–199, 1952.

250. HEWLETT, P. S., AND PLACKETT, R. L.: The relation between quantal and graded responses to drugs. Biometrics 12: 72–78, 1956.

251. HEWLETT, P. S., AND PLACKETT, R. L.: A unified theory for quantal responses to mixtures of drugs: non-interactive action. Biometrics 15: 591–596, 1959.

252. HEWLETT, P. S., AND PLACKETT, R. L.: A unified theory for quantal responses to mixtures of drugs: competitive interaction. Biometrics 20: 566–575, 1964.

253. HIATT, C. W., KAUFMAN, E., HELPRIN, J. J., AND BARON, S.: Inactivation of viruses by the photodynamic action of toluidine blue. J. Immunol. 84: 480–484, 1960.

254. HILL, A. V. The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curve. J. Physiol. 40: P4-P7, 1910.

255. HILL, A. V.: The combinations of haemoglobin with oxygen and with carbon monoxide. Biochem. J. 7: 471–480, 1913.

256. HIROSE, M., TAKEDA, E., NINOMIYA, T., KURODA, Y., AND NIYAO, M.: Synergistic inhibitory effects of dipyrimidamole and vincristine on the growth of human leukaemia cell lines. Br. J. Cancer 56: 413–417, 1987.

257. HOANG, T., HAMAN, A., GONCALVES, O., LETENDRE, F., MATHIEU, M., WONG, G. G., AND CLARK, S. C. Interleukin 1 enhances growth factor-dependent proliferation of the clonogenic cells in acute myeloblastic leukemia and of normal human primitive hemopoietic precursors. J. Exp. Med. 168: 463–474, 1988.

258. HOFMANN, J., DOPPLER, W., JAKOB, A., MALY, K., POSCH, L., ÜBERALL, F., AND GRUNICKE, H. H.: Enhancement of the antiproliferative effect of cis-diamminedichloroplatinum(II) and nitrogen mustard by inhibitors of protein kinase C. Int. J. Cancer 42: 382–388, 1988.

259. HORN, C. R., AND COCHRANE, G. M.: Management of asthma in general practice. Respir. Med. 83: 67–70, 1989.

260. HORREVORTS, A. M., DE RIDDER, C. M., POOT, M. C., DE JONGE, M. J. A., DEGENER, J. E., DZOLJIC-DANILOVIC, G., MICHEL, N. F., AND KERREBIJN, K. F.: Chequerboard titrations: the influence of the composition of serial dilutions of antibiotics on the fractional inhibitory index and fractional bactericidal concentrations. J. Antibiot. Chemother. 19: 119–125, 1987.

260a. HOWELL, S. B., HOM, D. K., SANGA, R., VICK, J. S., AND CHAN, T. C. K.: Dipyramidole enhancement of etoposide sensitivity. Cancer Res. 49: 4147–4153, 1989.

261. HUBBELL, H. R.: Synergistic antiproliferative effect of human interferons in combination with mismatched double-stranded RNA on human tumor cells. Int. J. Cancer 37: 359–365.

262. HUGHES, T. K., KASPAR, T. A., AND COPPENHAVER, D. H. Synergy of

UNITED THERAPEUTICS CORP., EX1034, page 43
UNITED THERAPEUTICS CORP. v. ACTELION PHARMACEUTICALS
U.S. PATENT 8,268,847

antiviral actions of TNF and IFN-γ: evidence for a major role of TNF-induced IFN-β. Antivir. Res. 10: 1–9, 1988.

263. HURWITZ, E., BUROWSKI, D., KASHI, R., HOLLANDER, N., AND HAIMOVICH, J.: A synergistic effect between anti-idiotype antibodies and anti-neoplastic drugs in the therapy of a murine B-cell tumor. Int. J. Cancer 37: 739–745, 1986.

264. HYMAN, A. M., AND FRANK, M. E.: Effects of binary taste stimuli on the neural activity of the hamster chorda tympani. J. Gen. Physiol. 76: 125–142, 1980.

265. IREGREN, A.: Effects on psychological test performance of workers exposed to a single solvent (toluene)—a comparison with the effects of exposure to a mixture of organic solvents. Neurobehav. Toxicol. Teratol. 4: 695–701, 1982.

266. ISIS-2 (SECOND INTERNATIONAL STUDY OF INFARCT SURVIVAL COLLABORATIVE GROUP: Randomised trial of intravenous streptokinase, oral aspirin, both, or neither among 17187 cases of suspected acute myocardial infarction: 1515–2. Lancet 2: 349–360, 1988.

267. ITO, M., HAYASHI, Y., TANABE, F., SHIGETA, S., AND KAWADE, Y.: Synergistic antiproliferative effect of interferon-β in combination with bleomycin or neocarzinostatin on HeLa cells in culture: additive effect when combined with adriamycin or mitomycin C. J. Interferon Res. 7: 419–425, 1987.

268. JACKSON, R. C.: Computer simulation of the effects of antimetabolites on metabolic pathways. *In* New Avenues in Developmental Cancer Chemotherapy, ed. by K. R. Harrap, and T. A. Connors, pp. 3–35, Academic Press, New York, 1987.

269. JACKSON, R. C., AND HARRAP, K. R.: Studies with a mathematical model of folate metabolism. Arch. Biochem. Biophys. 158: 827–841, 1973.

270. JACKSON, R. C., AND HARRAP, K. R.: Computer models of anticancer drug interaction. Pharmacol. Ther. 4: 245–280, 1979.

271. JENNETTE, K. W.: The role of metals in carcinogenesis: biochemistry and metabolism. Environ. Health Perspect. 40: 233–252, 1981.

272. JOHNSON, F. H., EYRING, H., AND KEARNS, W.: A quantitative theory of synergism and antagonism among diverse inhibitors, with special reference to sulfanilamide and urethane. Arch. Biochem. 3: 1–31, 1944.

273. JOHNSON, F. H., EYRING, H., AND WILLIAMS, R. W.: The nature of enzyme inhibitors in bacterial luminescence: sulfanilamide, urethane, temperature and pressure. J. Cell. Comp. Physiol. 20: 247–268, 1942.

274. JOHNSON, J. C., AND ATTANASIO, R.: Synergistic inhibition of anatid herpesvirus replication by acyclovir and phosphonocompounds. Intervirology 28: 89–99, 1987.

275. JUAN, H., SAMETZ, W., SARIA, A., AND PÖCH, G.: Eicosapentenoic acid inhibits vasoconstrictor-and noradrenaline-potentiating effects of neuropeptide Y in the isolated rabbit ear. J. Auton. Nerv. Syst. 22: 237–242, 1988.

276. KÄER, E., AND LOEWE, S.: Über Kombinationswirkungen. II. Mitteilung: Wirkungen von Diäthylbarbitursäure-Pyramidon-Gemisch. Arch. Exp. Pathol. Pharmacol. 114: 327–338, 1926.

277. KÄER, E., AND LOEWE, S.: Über Kombinationswirkungen. III. Mitteilung: Die Wirkungsvariationen in Veronal-Antipyrin-Gemischen. Arch. Exp. Path. Pharmakol. 114: 339–347, 1926.

278. KÄER, E., AND LOEWE, S.: Über Kombinationswirkungen. IV. Mitteilung: Die Wirkungsvariationen im Gemisch Veronal-Phenazetin. Arch. Exp. Path. Pharmakol. 116: 140–146, 1926.

279. KÄER, E., AND LOEWE, S.: Über Kombinationswirkungen. VI. Mitteilung: Die Wirkungsvariationen in Veronal-Azetysalizylsäuregemischen. Arch. Exp. Path. Pharmakol. 118: 108–114, 1926.

280. KAIBUCHI, K., TAKAI, Y., SAWAMURA, M., HOSHIJIMA, M., FUJIKURA, T., AND NISHIZUKA, Y.: Synergistic functions of protein phosphorylation and calcium mobilization in platelet activation. J. Biol. Chem. 258: 6701–6704, 1983.

281. KANO, Y., OHNUMA, T., OKANO, T., AND HOLLAND, J. F.: Effects of vincristine in combination with methotrexate and other antitumor agents in human acute lymphoblastic leukemia cells in culture. Cancer Res. 48: 351–356, 1988.

281a. KATAHARA, K., MIKAMI, H., OGIHARA, T., KOHARA, K., OTSUKA, A., KUMAHARA, Y., AND KHOSLA, M. C.: Synergism of intraventricular infusion and subpressor angiotensins in rats. Am. J. Physiol. 256: H1–H8, 1989.

282. KATAKAMI, Y., NAKAO, Y., KATAKAMI, N., KOIZUMI, T., OGAWA, R., YAMADA, H., TAKAI, Y., AND FUJITA, T.: Cooperative effects of tumor necrosis factor-α and 1,25-dihydroxyvitamin D_3 on growth inhibition, differentiation, and c-*myc* reduction in human promyelocyte leukemic cell line HL-60. Biochem. Biophys. Res. Commun. 152: 1151–1157, 1988.

283. KATZ, Y., COLE, F. S., AND STRUNK, R. C.: Synergism between γ interferon and lipopolysaccharide for synthesis of factor B, but not C2, in human fibroblasts. J. Exper. Med. 167: 1–14, 1988.

284. KELLEHER, C., MIYAUCHI, J., WONG, G., CLARK, S., MINDEN, M. D., AND MCCULLOCH, E. A.: Synergism between recombinant growth factors, GM-CSF and G-CSF, acting on the blast cells of acute myeloblastic leukemia. Blood 69: 1498–1503, 1987.

285. KELLY, C., AND RICE, J.: Monotone smoothing with application to dose response curves and the assessment of synergism. Biometrics. In press.

286. KERN, D. H., MORGAN, C. R., AND HILDEBRAND-ZANKI, S. U.: *In vitro* pharmacodynamics of 1-β-D-arabinofuranosylcytosine: synergy of antitumor activity with *cis*-diamminedichloroplatinum(II). Cancer Res. 48: 117–121, 1988.

287. KERRY, D. W., HAMILTON-MILLER, J. M. T., AND BRUMFITT, W.: Trimethoprim and rifampicin: *in vitro* activities separately and in combination. J. Antimicrob. Chemother. 1: 417–427, 1975.

288. KIM, H. C., AND RAESS, B. U.: Verapamil, diltiazem and nifedipine interactions with calmodulin stimulated (Ca^{2+} + Mg^{2+})-ATPase. Biochem. Pharmacol. 37: 917–920, 1988.

288a. KIMELMAN, D., AND KIRSCHNER, M.: Synergistic induction of mesoderm by FGF and TGFβ, and the identification of an mRNA coding for FGF in the early *Xenopus* embryo. Cell 51: 869–877, 1987.

289. KING, T. C., AND KROGSTAD, D. J.: Spectrophotometric assessment of dose-response curves for single antimicrobial agents and antimicrobial combinations. J. Infect. Dis. 147: 758–764, 1983.

290. KINN, A-C., AND LINDSKOG, M. Estrogens and phenylpropanolamine in combination for stress urinary incontinence in postmenopausal women. Urology 32: 273–280, 1988.

291. KIRSI, J. J., MCKERNAN, P. A., BURNS, N. J., III., NORTH, J. A., MURRAY, B. K., AND ROBINS, R. K.: Broad-spectrum synergistic antiviral activity of selenazofurin and ribavirin. Antimicrob. Agents Chemother. 26: 466–475, 1984.

292. KISSIN, I., BROWN, T., BRADLEY, E. L., JR., ROBINSON, A., AND CASSADY, J. L.: Diazepam-morphine hypnotic synergism in rats. Anesthesiology 70: 689–694, 1989.

293. KISSIN, I., KERR, C. R., AND SMITH, L. R.: Morphine-halothane interaction in rats. Anesthesiology 60: 553–561, 1984.

294. KISSIN, I., MASON, J. O., III., AND BRADLEY, E. L., JR.: Pentobarbital and thiopental anesthetic interactions with midazolam. Anesthesiology 67: 26–31, 1987.

295. KITAHARA, M., KATAKURA, R., MORI, T., SUZUKI, J., AND SASAKI, T.: Combined effect of ACNU and 5-FU on rat glioma cells in spheroids and monolayer culture. Int. J. Cancer 38: 215–222, 1986.

296. KITAHARA, M., KATAKURA, R., SUZUKI, J., AND SASAKI, T.: Experimental combination chemotherapy of ACNU and 5-FU against cultured glioma model (spheroid) and subcutaneous rat glioma. Int. J. Cancer 40: 557–563, 1987.

297. KLASTERSKY, J.: Combinations of antibiotics for therapy of severe infections in cancer patients. Eur. J. Cancer 15: 3–13, 1979.

298. KLASTERSKY, J., CAPPEL, R., AND DANEAU, D.: Clinical significance of in vitro synergism between antibiotics in gram-negative infections. Antibiot. Agents Chemother. 2: 470–475, 1972.

299. KLASTERSKY, J., MEUNIER-CARPENTER, F., AND PREVOST, J-M.: Significance of antimicrobial synergism for the outcome of gram negative sepsis. Am. J. Med. Sci. 273: 157–167, 1977.

300. KLASTERSKY, J., MEUNIER-CARPENTIER, F., PREVOST, J. M., AND STAQUET, M.: Synergism between amikacin and cefazolin against *Klebsiella:* in vitro studies and effect on the bactericidal activity of serum. J. Infect. Dis. 134: 271–276, 1976.

301. KLASTERSKY, J., AND ZINNER, S. H.: Synergistic combinations of antibiotics in gram-negative bacillary infections. Rev. Infect. Dis. 4: 294–301, 1982.

302. KODELL, R. L., AND POUNDS, J. G.: Characterization of the joint action of two chemicals in an in vitro test system. *In* Proc. Am. Stat. Assoc. Biopharm. Section, pp. 48–53, 1985.

303. KODELL, R. L., AND POUNDS, J. G.: Assessing the toxicity of mixtures of chemicals. *In* Statistical Methods in Toxicological Research, ed. by D. Krewski and C. Franklin, Cordon and Breach Science Publishers, New York, in press.

304. KOHNO, N., OHNUMA, T., KANEKO, M., AND HOLLAND, J. F.: Interactions of doxorubicin and *cis*-platin in squamous carcinoma cells in culture. Br. J. Cancer 58: 330–334, 1988.

305. KOIKE, K., NAKAHATA, T., TAKAGI, M., KOBAYASHI, T., ISHIGURO, A., TSUJI, K., NAGANUMA, K., OKANO, A., AKIYAMA, Y., AND AKABANE, T.: Synergism of BSF-2/interleukin 6 and interleukin 3 on development of multi-potential hemopoietic progenitors in serum-free culture. J. Exp. Med. 168: 879–890, 1988.

306. KÖNEMANN, H.: Fish toxicity tests with mixtures of more than two chemicals: a proposal for a quantitative approach and experimental results. Toxicology 19: 229–238, 1981.

306a. KOPIA, G. A., KOPACIEWCZ, L. J., OHLSTEIN, E. H., HOROHONICH, S., STORER, B. L., AND SHEBUSKI, R. T.: Combination of the thromboxane receptor antagonist, Salotroban (BM 13.17); SK & F 95587), with streptokinase: demonstration of thrombolytic synergy. J. Pharmacol. Exp. Ther. 250: 887–895, 1989.

307. KOSHLAND, D. E., JR.: The molecular basis for enzyme regulation. *In* The Enzymes, ed. by P. D. Boyer, Third Edition, Vol. 1, Academic Press, New York, pp. 341–396, 1970.

308. KREISMAN, H., FRANK, H., WOLKOVE, N., AND GENT, M.: Synergism between ipratropium and theophylline in asthma. Thorax 36: 387–391, 1981.

309. KRUNGKRAI, S. R., AND YUTHAVONG, Y.: The antimalarial action on *Plasmodium falciparum* of ginghaosu and artesunate in combination with agents which modulate oxidant stress. Trans. Roy. Soc. Trop. Med. Hygeine 81: 710–714, 1987.

310. KUSAMA, T., SUGIURA, N., KAI, M., AND YOSHIZAWA, Y.: Combined effects

of radiation and caffeine on embryonic development in mice. Radiat. Res. 117: 273–281, 1989.

311. KUSIMOTO, H., MAEHARA, Y., ANAI, H., KUSUMOTO, T., AND SUGIMACHI, K.: Potentiation of adriamycin cytotoxicity by dipyrimadole against HeLa cells *in vitro* and sarcoma 180 cells *in vivo*. Cancer Res. 48: 1208–1212, 1988.

312. LAM, G. K. Y.: The interaction of radiations of different LET. Phys. Med. Biol. 32: 1291–1309, 1987.

313. LAM, G. K. Y.: The survival response of a biological system to mixed radiations. Radiat. Res. 110: 232–243, 1987.

314. LAM, G. K. Y.: The combined effects of mixtures of ionizing radiations. J. Theor. Biol. 134: 531–546, 1988.

315. LAM. G. K. Y.: On the general validity of linear summation of dose equivalents for mixed radiations. Health Phys. 54: 57–61, 1988.

315a. LAMB, R. G., BORZELLECA, J. F., CONDIE, L. W., AND GENNINGS, C.: Toxic interactions between carbon tetrachloride and chloroform in cultured rat hepatocytes. Tox. Appl. Pharmacol. 101: 106–113, 1989.

316. LAPADULA, D. M., TILSON, H. A., CAMPBELL, G., AND ABOU-DONIA, M.: Neurotoxic effect of combined treatment of 2,5-hexandione and triethyllead chloride. J. Toxicol. Environ. Health 21: 483–492, 1987.

317. LAST, J. A., AND WARREN, D. L.: Synergistic interaction between nitrogen dioxide and respirable aerosols of sulfuric acid or sodium chloride on rat lungs. Toxicol. Appl. Pharmacol. 90: 34–42, 1987.

318. LEA, D. E.: Actions of Radiations on Living Cells. Cambridge University Press, Cambridge, 1955.

319. LEFER, A. M., MENTLEY, R., AND SUN, J-Z.: Potentiation of myocardial salvage by tissue type plasminogen activator in combination with a thromboxane synthetase inhibitor in ischemic cat myocardium. Circ. Res. 63: 621–627, 1988.

320. LEFF, A. R.: Endogenous regulation of bronchomotor tone. Am. Rev Respir. Dis. 137: 1198–1216, 1988.

321. LEFF, A. R., AND MUNOZ, N. M.: Cholinergic and *alpha* adrenergic augmentation of histamine-induced contraction of canine airway smooth muscle. J. Pharmacol. Exp. Ther. 218: 582–587, 1981.

322. LEITÃO, A.C., AND CARVALHO, R. E. S.: Synergistic killing of *Escherichia coli* K-12 by UV (254 pm) and H_2O_2. Int. J. Radiat. Biol. 53: 477–488, 1988.

323. LELIEVELD, P., SCOLES, M. A., BROWN, J. M., AND KALLMAN, R. F.: The effect of treatment in fractionated schedules with the combination of X-irradiation and six cytotoxic drugs on the R1F-1 tumor and normal mouse skin. Int. J. Radiat. Oncol. Biol. Phys. 11: 111–121, 1985.

323a. LEWTAS, J.: Genotoxicity of complex mixtures: strategies for the identification and comparative assessment of airborne mutagens and carcinogens from combustion sources. Fund. Appl. Toxicol. 10: 571–589, 1988.

324. LICHTENSTEIN, A. K., GANZ, T., SELSTED, M. E., AND LEHRER, R. I.: Synergistic cytolysis mediated by hydrogen peroxide combined with peptide defensins. Cell. Immunol. 114: 104–116, 1988.

325. LIGGINS, G. C., SCHELLENBERG, J-C., MANZAI, M., KITTERNAN, J. A., AND LEE, C-C. H.: Synergism of cortisol and thyrotropin-releasing hormone in lung maturation in fetal sheep. J. Appl. Physiol. 65: 1880–1884, 1988.

326. LIN, Y-C., HO, I-C., AND LEE, T-C.: Ethanol and acetaldehyde potentiate the clastogenicity of ultraviolet light, methyl methanesulfonate, mitomycin C and bleomycin in Chinese hamster ovary cells. Mutat. Res. 216: 93–99, 1989.

327. LINDSAY, D. A.: Fixed dose combination therapy in the treatment of asthma—the case against it. *In* Mechanisms in Asthma: Pharmacology, Physiology, and Management, ed. by C. L.Armour and J. L. Black, pp. 421–425, Alan R. Liss Inc., New York, 1988.

328. LINDSTRÖM, K.: Behavioral effects of long-term exposure to organic solvents. Acta Neurol. Scand. Suppl. 92 66: 131–141, 1982.

329. LIVINGSTON, G. K., AND DETHLEFSEN, L. A.: Effects of hyperthermia and X irradiation on sister chromatid exchange (SCE) frequency in Chinese hamster ovary (CHO) cells. Radiat. Res. 77: 512–520, 1977.

330. LLOYD, O. L.: Respiratory-cancer clustering associated with localised industrial air pollution. Lancet 1: 318–320, 1978.

331. LLOYD, O. L., AND GAILEY, F. A.: Techniques of low technology sampling of air pollution by metals: a comparison of concentrations and map patterns. Br. J. Indust. Med. 44: 494–504, 1987.

332. LOEWE, S.: Über Kombinationswirkungen. VIII. Mitteilung. Wirkungen von Diäthylbarbitursäure-p-Kresotinsäure-Codeingemischen. Arch. Exp. Pathol. Pharmakol. 120: 41–47, 1927.

333. LOEWE, S.: Die Mischarnei. Klin. Wochenschr. 6: 1077–1085, 1927.

334. LOEWE, S.: Die quantitativen Probleme der Pharmakologie. Ergebn. Physiol. 27: 47–187, 1928.

335. LOEWE, S.: The problem of synergism and antagonism of combined drugs. Arzneimittelforschung 3: 285–290, 1953.

336. LOEWE, S.: Isobols of dose-effect relations in the combination of pentylenetetrazole and phenobarbital. J. Pharmacol. Exp. Ther. 114: 185–191, 1955.

337. LOEWE, S.: Isobols of dose-effect relations in the combination of nikethamide (coramine) and phenobarbital. J. Pharmacol. Exp. Ther. 115: 6–15, 1955.

338. LOEWE, S.: Antagonisms and antagonists. Pharmacol. Rev. 9: 237–243, 1957.

339. LOEWE, S., ALDOUS, R. A., FOX, S. R., JOHNSON, D. G., AND PERKINS, W.: Isobols of dose-effect relations in the combination of pentylenetetrazole and trimethadone. J. Pharmacol. Exp. Ther. 113: 475–480, 1955.

340. LOEWE, S., KÄER, E., AND MUISCHNEK, H.: Uber Kombinationswirkungen. VII. Mitteilung.: Grundlagen der Prüfung von Drei-Pharmakamischungen.-Anwendung auf PhenazetinAzetylsalizylsäureCodeinemischungen. Arch. Exp. Path. Pharmakol. 120: 25–40, 1927.

341. LOEWE, S., AND MUISCHNEK, H.: Über Kombinationswirkungen. I. Mitteilung: Hilfsmittel der Fragestellung. Arch. Exp. Path. Pharmakol. 114: 313–326, 1926.

342. LOFTFIELD, R. B., AND EIGNER, E. A.: Molecular order of participation of inhibitors (or activators) in biological systems. Science 164: 305–307, 1969.

343. LONG, M. W., HUTCHINSON, R. J., GRAGOWSKI, L. L., HEFFNER, C. H., AND EMERSON, S. G.: Synergistic regulation of human megakaryocyte development. J. Clin. Invest. 82: 1779–1786, 1988.

344. LONG, M. W., SMOLEN, J. E., SZCZEPANSKI, P., AND BOXER, L. A.: Role of phorbol diesters in in vitro murine megakaryocyte colony formation. J. Clin. Invest. 74: 1686–1692, 1984.

345. LOOMIS, C. W., MILNE, S., AND CERVENKO, F. W.: A study of the interaction between clonidine and morphine on analgesia and blood pressure during continuous intrathecal infusion in the rat. Neuropharmacology 27: 191–197, 1988.

346. LORD, K. A.: The contact toxicity of a number of D.D.T. analogues and of four isomers of benzene hexachloride to *Macrosiphoniella sanborni* and *Oryzaephilus surinamensis*. Ann. Appl. Biol. 35: 505–526, 1948.

347. LUBIN, J. H., AND GAFFEY, W.: Relative risk models for assessing the joint effects of multiple factors. Am. J. Indust. Med. 13: 149–167, 1988.

348. MAGISTRETTI, P. J., AND SCHORDERET, M.: VIP and noradrenaline act synergistically to increase cyclic AMP in cerebral cortex. Nature 308: 280–282, 1984.

349. MANDEL, R., AND RYSER, H. J-P.: Mechanisms of synergism in the mutagenicity of cadmium and N-methyl-N-nitrosourea in *Salmonella typhimurium*: the effect of pH. Mutat. Res. 176: 1–10, 1987.

350. MANNINEN, O. (ed.): Combined Effects of Environmental Factors. Keskuspaino Central Publishing House, Helsinki, 1985.

351. MARKS, P. A., AND JOHNSON, A. B.: Relationship between the age of human erythrocytes and their osmotic resistance: a basis for separating young and old erythrocytes. J. Clin. Invest. 37: 1542–1548.

352. MARTH, C., DAXENBICHLER, G., AND DAPUNT, O.: Synergistic antiproliferative effect of human recombinant interferons and retinoic acid. J. Natl. Cancer Inst. 77: 1197–1202, 1986.

353. MARTH, C., KIRCHEBNER, P., AND DAXENBICHLER, G.: The role of polyamines in interferon and retinoic acid mediated synergistic antiproliferative action. Cancer Lett. 44: 55–59, 1989.

354. MARTINEZ, S., LOOKER, D. L., BERENS, R. L., AND MARR, J. J.: The synergistic action of pyrazolopyrimidines and pentavalent antimony against *Leishmania donovani* and *L. braziliensis*. Am. J. Trop. Med. Hyg. 39: 250–255, 1988.

355. MARUYAMA, Y., DARWAZEH, N. B., AND MAGURA, C.: Increased ^{252}Cf therapeutic advantage for an advanced murine lymphoma (LSA) growing *in vivo*. Br. J. Radiol. 61: 312–316, 1988.

356. MASUDA, Y., UTSUI, Y., SHIRAISHI, Y., KARASAWA, T., YOSHIDA, K., AND SHIMUZU, U.: Evidence for a synergistic interaction between phenytoin and phenobarbital in experimental animals. J. Pharmacol. Exper. Ther. 217: 805–811, 1981.

357. MATHER, K.: The design and significance of synergic action tests. J. Hyg. 40: 513–531, 1940.

358. MAUGH, T. H.: Just how hazardous are dumps? Science 215: 490–493, 1982.

359. MAY, F. E., STEWART, R. B., AND CLUFF, L. E.: Drug interactions and multiple drug administration. Clin. Pharmacol. Ther. 22: 322–328, 1977.

360. MCCABE, W. R.: Clinical use of combinations of antimicrobial agents. Antimicrob. Agents Chemother. 225–233, 1967.

361. MCNIECE, I. K., ROBINSON, B. E., AND QUESENBERRY, P. J.: Stimulation of murine colony-forming cells with high proliferative potential by the combination of GM-CSF and CSF-1. Blood 72: 191–195, 1988.

362. MITCHELL, C. L.: The design and analysis of experiments for the assessment of drug interactions. Ann. N.Y. Acad. Sci. 281: 118–135, 1976.

363. MITCHISON, D. A.: Combined antimicrobial treatment in tuberculosis and in non-tuberculous infections. *In* Combined Antimicrobial Therapy, ed. by W. Brumfitt, L. Cuscio, and L. Silvestri, pp. 45–58, Martinus Nyhoff, The Hague, 1979.

364. MIZUSHIMA, Y. SAITOH, M., OGATA, M., KOSAKI, H., TATSUMI, Y., KIYOTAHI, C., HAMAOKA, T., AND FUJIWARA, H.: Thymic stroma-derived T cell growth factor (TSTGF). IV. Capacity of TSTGF to promote the growth of L3T4-Lyt2-thymocytes by synergy with phorbol myristate acetate or various IL. J. Immunol. 142: 1195–1202, 1989.

365. MOMPARLER, R. L.: *In vitro* systems for evaluation of combination therapy. Pharmacol. Ther. 8: 21–35, 1980.

366. MOORE, G. J.: Methods for analyzing and interpreting cooperativity in dose-response curves. I. Antagonistic effects on angiotensin receptors in smooth muscle. Gen. Pharmacol. 20: 193–198, 1989.

367. MOORE, G. J.: Methods for analyzing and interpreting cooperativity in dose-response curves. II. Partial agonists acting on muscarinic receptors in smooth muscle. Gen. Pharmacol. 20: 199–203, 1989.

368. MORAN, D. M., KERN, E. R., AND OVERALL, J. C., JR.: Synergism between recombinant human interferon and nucleoside antiviral agents against herpes simplex virus: examination with an automated microtiter plate assay. J. Infect. Dis. 151: 1116–1122, 1985.
369. MOSER, M.: Relative efficacy of, and some adverse reactions to, different antihypertensive regimens. Am. J. Cardiol. 63: 2B-7B, 1989.
370. MÜLLER, W-U., AND STREFFER, C.: Risk to preimplantation mouse embryos of combinations of heavy metals and radiation. Int. J. Radiat. Biol. 51: 997–1006, 1987.
371. MÜLLER, W-U., STREFFER C., AND WURM, R.: Supraadditive formation of micronuclei in preimplantation mouse embryos in vitro after combined treatment with X-rays and caffeine. Terat. Carcinogen. Mutat. 5: 123–131, 1985.
372. MUROHASHI, I., NAGATA, K., SUZUKI, T., MARUYAMA, Y., AND NARA, N.: Effects of recombinant G-CSF and GM-CSF on the growth in methylcellulose and suspension of the blast cells in acute myeloblastic leukemia. Leuk. Res. 12: 433–440, 1988.
373. MURRAY, P. R.: Activity of cefotaxime-aminoglycoside combinations against aminoglycoside-resistant *Pseudomonas*. Antibiot. Agents Chemother. 17: 474–476, 1980.
374. MYERS, R. H.: Response Surface Methodology, Allyn and Bacon Inc., Boston, 1971.
375. MYERS, R. H., AND CARTER, W. H., JR.: Response surface techniques for dual response systems. Technometrics 15: 301–317, 1973.
376. NELDER, J. A., AND MEAD, R.: A simplex method for function minimization. Comput. J. 7: 308–313, 1964.
377. NIAS, A. H. W.: Radiation and platinum drug interaction. Int. J. Radiat. Biol. 48: 297–314, 1985.
378. NICHOLS, A. C.: Bactericidal action of streptomycin-penicillin mixtures *in vitro*. Proc. Soc. Exp. Biol. Med. 69: 477–478, 1948.
379. NICHOLS, W. W., MEHTA, J. L., THOMPSON, L., AND DONNELLY, W. H.: Synergistic effects of LTC_4 and TxA_2 on coronary flow and myocardial function. Am. J. Physiol. 255: H153-H159, 1988.
380. NICHOLSON, S. A., ADRIAN, T. E., GILLHAM, S., JONES, M. T., AND BLOOM, S. R.: Effects of hypothalamic neuropeptides on corticotrophin release from quarters of rat anterior pituitary gland *in vitro*. J. Endocrin. 100: 219–226, 1984.
381. NISHIZUKA, Y.: The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 308: 693–698, 1984.
382. NISHIZUKA, Y., TAKAI, Y., KISHIMOTO, A., KIKKAWA, U., AND KAIBUCHI, K.: Phospholipid turnover in hormone action. Rec. Progr. Hormone Res. 40: 301–345, 1984.
383. NORBERG, L., AND WAHLSTRÖM, G.: Interactions between hexobarbital and thiopental in male rats evaluated with an anaesthesia threshold. Acta Pharmacol. Toxicol. 58: 96–104, 1986.
384. NORBERG, L., AND WAHLSTRÖM, G.: Anaesthetic effects of flurazepam alone and in combination with thiopental or hexobarbital evaluated with an EEG-threshold method in male rats. Arch. Int. Pharmacodyn. Ther. 292: 45–57, 1988.
385. NORDEN, C. W.: Problems in determination of antibiotic synergism in vitro. Rev. Infect. Dis. 4: 276–281, 1982.
386. NORMAN, L. J., AND CHALLIS, J. R. G.: Synergism between systemic corticotropin-releasing factor and arginine vasopressin on adrenocorticotropin release *in vivo* varies as a function of gestational age in the ovine fetus. Endocrinology 120: 1052–1058, 1987.
387. NOSEDA, A., BERENS, M. E., WHITE, J. G., AND MODEST, E. J.: *In vitro* antiproliferative activity of combinations of ether lipid analogues and DNA-interactive agents against human tumor cells. Cancer Res. 48: 1788–1791, 1988.
388. ODDS, F. C.: Interactions among amphotericin B, 5-fluorocytosine, ketoconazole, and miconazole against pathogenic fungi in vitro. Antibiot. Agents Chemother. 22: 763–770, 1982.
389. ÖDKVIST, L. N., LARSBY, B., FREDERICKSON, J. M. F., LIEDGREN, S. R. C., AND THAM, R.: Vestibular and oculomotor disturbances caused by industrial solvents. J. Otolaryngol. 9: 53–59, 1980.
390. OKADA, A., AND MANNINEN, O. (eds.): Recent Advances in Researches on the Combined Effects of Environmental Factors. Kyoei Co., Kanazawa, 1987.
391. OKUSAWA, S., GELFAND, J. A., IKEJIMA, T., CONNOLY, R. J., AND DINARELLO, C. A.: Interleukin 1 induces a shock-like state in rabbits. Synergism with tumor necrosis factor and the effect of cyclooxygenase inhibition. J. Clin. Invest. 81: 1162–1172, 1988.
392. ONODA, J. M., JACOBS, J. R., TAYLOR, J. D., SLOANE, B. F., AND HONN, K. V.: Cisplatin and nifedipine: synergistic cytotoxicity against murine solid tumors and their metastases. Cancer Lett. 30: 181–188, 1986.
393. OTANI, S., MATSUI-YUASA, I., MIMURA-SHIMAZU, Y., AND MORISAWA, S.: Synergistic stimulation of S-adenosylmethionine decarboxylase activity by Ca^{2+} ionophore A23187, cholera toxin and 1-oleoyl-2-acetylglycerol. Eur. J. Biochem. 171: 509–513, 1988.
394. OWEN-SCHAUB, L. B., GUTTERMAN, J. V., AND GRIMM, E. A.: Synergy of tumor necrosis factor α and interleukin 2 in the activation of human cytotoxic lymphocytes: effect of tumor necrosis factor and interleukin 2 in the generation of human lymphokine-activated killer cell cytotoxicity. Cancer Res. 48: 788–792, 1988.
395. OZENNE, G., MOREL, A., MENARD, J. F., THAUVIN, C., SAMAIN, J. P., AND LEMELAND, J. F.: Susceptibility of *Mycobacterium avium* complex to various two-drug combinations of antituberculous agents. Am. Rev. Respir. Dis. 138: 878–881, 1988.
396. PACHTER, J. A., LAW, G. J., AND DANNIES, P. S.: TRH and BAY K8644 synergistically stimulate prolactin release but not $^{45}Ca^{2+}$ uptake. Am. J. Physiol. 255: C633-C640, 1988.
397. PALATINI, P.: The interaction between full and partial inhibitors acting on a single enzyme. Mol. Pharmacol. 24: 30–41, 1983.
398. PATERSON, A. R. P., AND MORIWAKI, A.: Combination chemotherapy: synergistic inhibition of lymphoma L5178Y cells in culture and *in vivo* with 6-mercaptopurine and 6-(methylmercapto) purine ribonucleoside. Cancer Res. 29: 681–686, 1969.
399. PELUS, L. M., OTTMAN, O. G., AND NOCKA, K. H.: Synergistic inhibition of human marrow granulocyte-macrophage progenitor cells by prostaglandin E and recombinant interferon-α, -β, and -γ and an effect mediated by tumor necrosis factor. J. Immunol. 140: 478–484, 1988.
400. PEREA, E. J., CLAVIJO, E., NOGALES, M. C., AND LUQUE, I. G.: Interaction of aminoglycosides and cephalosporins against *Pseudomonas aeroginosa*. Correlation between interaction index and killing curve. J. Antimicrob. Chemother. 22: 175–183, 1988.
401. PETO, R.: Carcinogenic effects of chronic exposure to very low levels of toxic substances. Environ. Health Perspect. 22: 155–159, 1978.
402. PFEILSCHIFTER, J., PIGNAT, N., VOSBECK, K., AND MÄRKI, F.: Interleukin 1 and tumor necrosis factor synergistically stimulate prostaglandin synthesis and phospholipase A2 release from rat renal mesangial cells. Biochem. Biophys. Res. Comm. 159: 385–394, 1989.
403. PHILLIPS, G. D., AND HOLGATE, S. T.: Interaction of inhaled LTC_4 with histamine and $P6D_2$ on airway caliber in asthma. J. Appl. Physiol. 66: 304–312, 1989.
404. PLAA, G. L., AND HEWITT, W. R.: Methodological approaches for interaction studies: potentiation of haloalkane-induced hepatotoxicity. *In* Workshop on the Combined Effects of Xenobiotics, Ottowa 22–23 June, 1981, ed. by H. F. Stich, H. W. Leung, and J. R. Roberts, pp. 67–96, National Research Council of Canada Publication No. 18978, 1982.
405. PLACKETT, R. L., AND HEWLETT, P. S.: Statistical aspects of the independent joint action of poisons, particularly insecticides. 1. The toxicity of a mixture of poisons. Ann. Appl. Biol. 35: 347–358, 1948.
406. PLACKETT, R. L., AND HEWLETT, P. S.: Quantal responses to mixtures of poisons. J. R. Stat. Soc. B 14: 141–163, 1952.
407. PLACKETT, R. L., AND HEWLETT, P. S.: A unified theory for quantal responses to mixtures of drugs: the fitting to data of certain models for two non-interactive drugs with complete positive correlation of tolerances. Biometrics 19: 517–531, 1963.
406. PLACKETT, R. L., AND HEWLETT, P. S.: A comparison of two approaches to the construction of models for quantal responses to mixtures of drugs. Biometrics 23: 27–44, 1967.
409. PÖCH, G.: Quantitative Ermittlung potenzierender oder hemmender Kombinationswirkungen gleichsinnigwirkender Pharmaka. Arzneimittelforschung 31: 1135–11450, 1981.
410. PÖCH, G.: The confusion about additive combinations. Trends Pharmacol. Sci. 2: 256–257, 1981.
411. PÖCH, G.: Clinical trials with combinations of drugs. *In* Multinational Registration of Drugs, ed. by H. P. Kuemmerle and H. Jaeger, Prog. Clin. Pharmacol. 6: 53–61, 1984.
412. PÖCH G., AND HOLZMANN, S.: Quantitative estimation of overadditive and underadditive drug effects by means of theoretical, additive dose-response curves. J. Pharmacol. Methods 4: 179–188, 1980.
413. PÖCH, G., AND REIFFENSTEIN, R. J.: Underadditive isoboles may represent independent combined action rather than antagonism (abstract). Naunyn-Schmiedeberg's Arch. Pharmacol. Suppl. 338: 236, 1988.
414. POTPIK, W., AND INGLOT, A. D.: Interactions of interferons and transforming growth factors during clonal growth of mouse or human cells in soft agar and in mice. Int. J. Cancer. 40: 108–113, 1987.
415. POTMESIL, M., ISRAEL, M., KIRSCHENBAUM, S., BOWEN, J., AND SILBER, R.: Effects of N-trifluoroacetyladriamycin14-Q-hemiadipate and radiation on L1210 cells. Radiat. Res. 105: 147–157, 1986.
415a. RAHIMTOOLA, S. H.: The pharmacologic treatment of chronic congestive heart failure. Circulation 80: 693–699, 1989.
416. RAINE, A. E. G., AND LEDINGHAM, J. G. G.: Principles of therapy for hypertension. *In* Scientific Foundations of Cardiology, ed. by P. Sleight and J. V. Jones, pp. 191–198, William Heinemann, London, 1983.
417. REDDY, S., KELLY, D., COCHINEAS, C., AND GYÖRY, A. Z.: Additive and synergistic interaction of atrial natriuretic peptide and volume expansion. Am. J. Physiol. 255: F66-F73, 1988.
418. REIF, A. E.: Synergism in carcinogenesis. J. Natl. Cancer Inst. 73: 25–39, 1984.
419. RENNICK, D., JACKSON, J., MOULDS, C., LEE, F., AND YANG, G.: IL-3 and stromal cell-derived factor synergistically stimulate the growth of pre-B cell lines cloned from long-term lymphoid bone marrow cultures. J. Immunol. 142: 161–166, 1989.
420. RILEY, P. A., STEVENS, M. T., MATHER, M. E., AND EADY, R. P.: Synergistic activity of sodium cromoglycate and β_2-stimulants in rat lung anaphylaxis. Eur. J. Pharmacol. 141: 143–147, 1987.
421. RIVIER, C., AND VALE, W.: Interaction of cortocotropin-releasing factor

(CRF) and arginine vasopressin (AVP) on ACTH secretion *in vivo*. Endocrinology **113**: 939–942, 1983.

422. ROBINSON, L. A., BRAIMBRIDHE, M. W., AND HEARSE, D. J.: Enhanced myocardial protection with high-energy phosphates in St.Thomas' Hospital cardioplegic solution. Synergism of adenosine triphosphate and creatine phosphate. J. Thorac. Cardiovasc. Surg. **93**: 415–427, 1987.
423. ROTHMAN, K. J.: The estimation of synergy or antagonism. Am. J. Epidemiol. **103**: 506–511, 1976.
424. ROTHMAN, K. J., GREENLAND, S., AND WALKER, A. M.: Concepts of interaction. Am. J. Epidemiol. **112**: 467–470, 1980.
425. ROTHSTEIN, J. L., AND SCHREIBER, H.: Synergy between tumor necrosis factor and bacterial products causes hemorrhagic necrosis and lethal shock in normal mice. Proc. Natl. Acad. Sci. **85**: 607–611, 1988.
426. ROZENGURT, E.: Early signals in the mitogenic response. Science **234**: 161–166, 1986.
427. ROZENGURT, E.: Signal transduction pathways in mitogenesis. Br. Med. Bull. **45**: 515–528, 1989.
428. ROZENGURT, E., AND MENDOZA, S. A.: Synergistic signals in mitogenesis: role of ion fluxes, cyclic nucleotides and protein kinase C in Swiss 3T3 cells. J. Cell Sci. (suppl 2) **3**: 229–242, 1985.
429. RUBIN, J. E., CHENOWETH, D. E., AND CATHERWOOD, B. D.: 1,25-Dihydroxyvitamin D_3 and adenosine 3′,5′-monophosphate synergistically promote differentiation of a monocyte cell line. Endocrinology **118**: 2540–2545, 1986.
430. RUNGBY, J.: Silver-induced lipid peroxidation in mice: interactions with selenium and nickel. Toxicology **45**: 135–142, 1987.
431. RUTHSATZ, M., AND NEUMANN, H-G.: Synergistic effects on the initiation of rat liver tumors by *trans*-4-acetylaminostilbene and 2-acetylaminofluorene, studied at the level of DNA adduct formation. Carcinogenesis **9**: 265–269, 1988.
432. SAARIALHO-KERE, U.: Psychomotor, respiratory and neuroendocrinological effects of nalbuphine and haloperidol, alone and in combination, in healthy subjects. Br. J. Clin. Pharmacol. **26**: 79–87, 1988.
433. SASATH, L. D., ELDER, H. A., MCCALL, C. E., AND FINLAND, M.: Synergistic combinations of penicillins in the treatment of bacteriuria. N Engl. J. Med. **277**: 232–238, 1967.
434. SAKAMOTO, K., KOMATSU, K., AND MATSOMOTO, Y.: The *in vitro* and *in vivo* antitumor effects of pepleomycin alone or in combination with radiation. Radiat. Res. **102**: 224–231, 1985.
435. SARACCI, R.: Asbestos and lung cancer: an analysis of the epidemiological evidence on the asbestos-smoking interaction. Int. J. Cancer **20**: 323–331, 1977.
436. SARACCI, R.: Interaction and synergism. Am. J. Epidemiol. **112**: 465–466, 1980.
437. SATO, A., AND CORY, J. G.: Evaluation of combinations of drugs that inhibit Ehrlich tumor cell ribonucleotide reductase. Cancer Res. **412**: 1637–1641, 1981.
438. SATO, K., FUJII, Y., KASONO, K., OZAWA, M., IMAMURA, H., KANAJI, Y., KUROSAWA, H., TSUSHIMA, T., AND SHIZOME, K.: Parathyroid hormone-related protein and interleukin-1 α synergistically stimulate bone resorption *in vitro* and increase the serum calcium concentration in mice *in vivo*. Endocrinology **124**: 2172–2178, 1989.
439. SCAF, A. H. J.: Statistical analysis of isoboles. Arch. Int. Pharmacodyn. **208**: 138–165, 1974.
440. SCHELLENSERG, J-C., LIGGINS, G. C., MANZAI, M., KITTERMAN, J. A., AND LEE, C. C. H.: Synergistic hormonal effects on lung maturation in fetal sheep. J. Appl. Physiol. **65**: 94–100, 1988.
441. SCHILLER, J. H., GROVEMAN, D. S., SCHMID, S. M., WILLSON, J. K. V., CUMMINGS, K. B., AND BORDEN, E. C.: Synergistic antiproliferative effects of human recombinant α54-or β-$_{ser}$-interferon with γ-interferon on human cell lines of various histogenesis. Cancer Res. **46**: 483–488, 1986.
442. SCHINAZI, R. F., CHOU, T-C., SCOTT, R. T., YAO, X., AND NAHMIAS, A. J.: Delayed treatment with combinations of antiviral drugs in mice infected with herpes simplex virus and application of the median effect method of analysis. Antibiot. Agents Chemother. **30**: 491–498, 1986.
443. SCOTT, B. R.: Theoretical models for estimating dose-effect relationships after combined exposure to cytotoxicants. Bull. Math. Biol. **45**: 323–345, 1983.
444. SCOTT, H. V., RIECKMANN, K. H., AND O'SULLIVAN, W. J.: Synergistic antimalarial activity of dapsone/dihydrofolate reductase inhibitors and the interaction of antifol, antipyrimidine and antipurine combinations against *Plasmodium falciparum in vitro*. Trans. R. Soc. Trop. Med. Hyg. **81**: 715–721, 1987.
445. SEGEL, I. H.: Enzyme Kinetics, John Wiley, New York, 1975.
446. SELIKOFF, I. J., AND HAMMOND, E. C.: Multiple risk factors in environmental cancer. *In* Persons at High Risk of Cancer, ed. by J. F. Fraumeni, Jr., pp. 467–484, Academic Press, New York, 1975.
447. SELIKOFF, I. J., HAMMOND, E. C., AND CHURG, J.: Asbestos exposure, smoking, and neoplasia. J. Am. Med. Assoc. **204**: 104–110, 1968.
448. SELIKOFF, I. J., SEIDMAN, H., AND HAMMOND, E. C.: Mortality effects of cigarette smoking among amosite asbestos factory workers. J. Natl. Cancer Inst. **65**: 507–513, 1980.
449. SHAIKH, N. A., OWEN, A. M., GHILCHIK, M. W., AND BRAUNSBERGER, H.: Actions of medroxyprogesterone acetate on the efficacy of cytotoxic drugs: studies with human breast cancer cells in culture. Int. J. Cancer **43**: 458–463, 1989.
450. SHAPIRO, G. G., MCPHILLIPS, J. J., SMITH, K., FURUKAWA, C. T., PIERSON, W. E., AND BIERMAN, C. W.: Effectiveness of terbutaline and theophylline alone and in combination in exercise-induced bronchospasm. Pediatrics **67**: 508–513, 1981.
451. SHELTON, D. W., AND WEBER, L. J.: Quantification of the joint effect of hepatotoxic agents: Evaluation of a theoretical model in mice. Environ. Res. **26**: 33–41, 1981.
452. SHENFIELD, G. M.: Combination bronchodilator therapy. Drugs **24**: 414–439, 1982.
453. SIEMANN, D. W., AND KENG, P. C.: Responses of tumor cell subpopulations to single modality and combined modality therapies. Natl. Cancer Inst. Monographs **6**: 101–105, 1988.
454. SIEMANN, D. W., MADDISON, K., WOLF, K., HILL, S. A., AND KENG, P. C.: *In vivo* interaction between radiation and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosoures in the absence or presence of misonidazole in mice. Cancer Res. **45**: 198–202, 1985.
455. SIEMATYCKI, J, AND THOMAS, D. C.: Biological models and statistical interactions: an example from multistage carcinogenesis Int. J. Epidemiol. **10**: 383–387, 1981.
455a. SIESS, W., AND LAPETINA, E. G.: Platelet aggregation induced by α_2-adrenoceptor and protein kinase C activation. A novel synergism. Biochem. J. **263**: 377–385, 1989.
455b. SILKWORTH, J. B., CUTLER, D. S., ANTRIM, L., HOUSTON, D., TOMASONIS, C., AND KAMINISKY, L. S.: Teratology of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in a complex environmental mixture from the Love Canal. Fund. App. Toxicol. **13**: 1–15, 1989.
456. SILKWORTH, J. B., CUTLER, D. S., AND SACK, G.: Immunotoxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in a complex environmental mixture from the Love Canal. Fund. Appl. Toxicol. **12**: 303–312, 1989.
457. SIM, I. S., AND CERRUTI, R. L.: Recombinant interferons alpha and gamma: comparative antiviral activity and synergistic interaction in encephalomyocaridtis virus infection of mice. Antiviral Res. **8**: 209–221, 1987.
458. SIMMONS, J. E., DEMARINI, D. M., AND BERMAN, E.: Lethality and hepatotoxicity of complex waste mixtures. Environ. Res. **46**: 74–85, 1988.
459. SIMON, E. R., AND TOPPER, Y. J.: Fractionation of human erythrocytes on the basis of their age. Nature **180**: 1211–1212, 1957.
460. SIMONE, J. V.: Childhood leukemia as a model for cancer research. Cancer Res. **39**: 4301–4307, 1979.
460a. SLACK, J. M. W.: Peptide regulatory factors in embryonic development. Lancet **1**: 1312–1315, 1989.
461. SLAPAK, M.: Triple and quadruple immunosuppresive therapy in organ transplantation. Lancet **2**: 958–960, 1987.
462. SMITH, B. T., AND SABRY, K.: Glucocorticoid-thyroid synergism in lung maturation: a mechanism involving epithelial-mesenchymal interaction. Proc. Nat. Acad. Sci. **80**: 1951–1954, 1983.
463. SMITH, E. F., III., AND EGAN, J. W.: Comparison of the effects of a thromboxane synthase inhibitor or prostacyclin in combination with a phosphodiesterase inhibitor for prevention of experimental thrombosis and sudden death in rabbits. J. Pharmacol. Exp. Ther. **241**: 855–860, 1987.
464. SMITH, G. H., WILLIAMS, F. L. R., AND LLOYD, O. L.: Respiratory cancer and air pollution from iron foundries in a Scottish town: an epidemiological and environmental study. Br. J. Indust. Med. **44**: 795–802, 1987.
464a. SMITH, M. S., BRIAN, E. L., DECLERCQ, E., AND PAGANO, J. S.: Susceptibility of human immunodeficiency virus type 1 replication in vitro to acyclic adenosine analogs and synergy of the analogs with 3′-azido-3′-deoxythymidine. Antibiot. Agents Chemother. **33**: 1482–1486, 1989.
465. SMITH, N. T.: Use of isobolograms in anesthesia. Anesth. Analg. **45**: 467–473, 1966.
466. SMITH, S. E., AND HERXHEIMER, A.: Toxicity of ethanolbarbiturate mixtures. J. Pharm. Pharmacol. **21**: 869–870, 1969.
467. SMITH, T. W., AND BRAUNWALD, E.: The management of heart failure. *In* Heart Disease, ed. by E. Braunwald, pp. 509–570, W. B. Saunders, Philadelphia, 1980.
468. SNAMESKI, M.: Über Kombinationswirkungen V. Mitteilung: Die Säugerherz Wirkung von Strophanthus-Digitalis-Gemischen Arch. Exp. Path. Pharmakol. **116**: 147–157, 1926.
469. SOBRERO, A. F., AND BERTINO, J. R.: Sequence-dependent synergism between dichloromethotrexate and 5-fluorouracil in a human colon carcinoma line. Cancer Res. **43**: 4011–4013, 1983.
470. SÖDERSTEN, P., ENEROTH, P., AND HANSSON, T.: Oestradiol synergizes with 5α-dihydrotestoterone or 3α- but not 3β-androstandiol in inducing sexual behaviour in castrated rats. J. Endocrinol. **119**: 461–465, 1988.
471. SOIKE, K. F., EPPSTEIN, D. A., GLOFF, C. A., CANTRELL, C., CHOU, T-C., AND GERONE, P. J.: Effect of 9(1,3-dihydroxy-2-propoxymethyl) guanine and recombinant human β interferon alone and in combination on simian varicella virus infection in monkeys. J. Infect. Dis. **156**: 607–614, 1987.
472. SOLANA, R. P., CARTER, W. H., JR., WILSON, J. D., CHINCHILLI, V. M., AND CARCHMAN, R. A.: Qualitative evaluation of sister chromatid exchanges elicited by combinations of genotoxic compounds using response surface methodology. J. Am. Coll. Toxicol. **7**: 975–986, 1988.
473. SOLANA, R. P., CHINCHILLI, V. M., CARTER, W. H., JR., WILSON, J. D.,

AND CARCHMAN, R. A.: The evaluation of biological interactions using response surface methodology. Cell Biol. Toxicol. 3: 263–277, 1987.

474. SOLANA, R. P., CHINCHILLI, V. M., WILSON, J., CARTER, W. H., JR., AND CARCHMAN, R. A.: Estimation and analysis of the concentration-response surfaces associated with multiple-agent combinations. Toxicol. Appl. Pharmacol. 85: 231–238, 1986.

475. SPADA, C. S., WOODWARD, D. F., HAWLEY, S. B., NIEVES, A. L., WILLIAMS, L. S., AND FELDMANN, B. J.: Synergistic effects of LTB_4 and LTD_4 on leukocyte emigration into the guinea pig conjunctiva. Am. J. Pathol. 130: 354–368, 1988.

476. SPENDLEY, W., HEXT, G. R., AND HIMSWORTH, F. R.: Sequential application of simplex designs in optimisation and evolutionary operation. Technometrics 4: 441–461, 1962.

477. STABLEIN, D. M., CARTER, W. H., JR., AND WAMPLER, G. L.: Survival analysis of drug combinations using a hazards model with time-dependent covariates. Biometrics 36: 537–546, 1980.

477a. STAMLER, J. S., VAUGHAN, D. E., AND LOSCALZO, J.: Synergistic disaggregation of platelets by tissue-type plasminogen activator, prostaglandin E_1, and nitroglycerin. Circulation Res. 65: 796–804, 1989.

478. STANISWALIS, J. G., AND MCCRADY, C. W.: The use of kernel estimators in describing human T lymphocyte proliferation induced by phorbol esters and Ca^{2+}+ionophore. J. Am. Coll. Toxicol. 7: 939–951, 1988.

479. STEEL, G. G.: Terminology in the description of drug-radiation interactions. Int. J. Radiat. Oncol. Biol. Phys. 5: 1145–1150, 1979.

480. STEEL, G. G., AND PECKHAM, M. J.: Exploitable mechanisms in combined radiotherapy-chemotherapy the concept of additivity. Int. J. Radiat. Oncol. Biol. Phys. 5: 85–91, 1979.

481. STEEN, V. M., AND HOLMSEN, H.: Synergism between thrombin and epinephrine in human platelets: different dose-response relationships for aggregation and dense granule secretion. Thromb. Res. 54: 680–683, 1985.

482. STEEN, V. M., TYSNES, O-B., AND HOLMSEN, H.: Synergism between thrombin and adrenaline (epinephrine) in human platelets. Marked potentiation of inositol phospholipid metabolism. Biochem. J. 253: 581–586, 1988.

483. STICH, H. F., LEUNG, H. W., AND ROBERTS, J. R. (eds.) Workshop on the Combined Effects of Xenobiotics, Ottowa, 22–23 June, 1981. National Research Council of Canada. Publication No. 18978, 1982.

484. STOCKLEY, I. H.: Drug Interactions, Blackwell Scientific Publications, Oxford, 1981.

485. STREFFER, C., AND MÜLLER, W-U.: Radiation risk from combined exposures to ionizing radiations and chemicals. Adv. Radiat. Biol. 11: 173–210, 1984.

486. STREFFER, C., AND MÜLLER, W-U.: Dose-effect relationships and general mechanisms of combined exposures. Int. J. Radiat. Biol. 51: 961–969, 1987.

487. STRINGER, A.: The insecticidal action of some D.D.T. analogues and chlorinated (4chlorophenyl)-ethanes. Ann. Appl. Biol. 36: 206–212, 1949.

488. STRUWE, G., AND WENNBERG, A.: Psychiatric and neurological symptoms in workers occupationally exposed to organic solvents-results in a differential epidemiological study. Acta Psychiat. Scand. (suppl. 303) 67: 68–80, 1983.

489. STÜRZEBECHER, S., AND WITT, W.: The PCI_2-analogue Iloprost and the TXA_2receptor antagonist Sulotroban synergistically inhibit TXA_2 -dependent platelet activation. Prostaglandins 36: 751–760, 1988.

490. SUNDERMAN, F. W., BIBEAU, L. M., AND REID, M. C.: Synergistic induction of microsomal heme oxygenase activity in rat liver and kidney by diethyldithiocarbamate and nickel chloride. Toxicol. Appl. Pharmacol. 71: 436–444, 1983.

491. SYRACUSE, K. C., AND GRECO, W.R.: Comparison between the method of Chou and Talalay and a new method for the assessment of the combined effects of drugs: a Monte Carlo simulation study. Proc. Am. Stat. Assoc. Biopharmacol. Section, 127–132, 1986.

492. SZUMIEL, I., AND NIAS, A. H. W.: Isobologram analysis of the combined effects of anti-tumour platinum complexes and ionizing radiation on mammalian cells. Br. J. Cancer 42: 292–296, 1980.

493. TANIZAWA, A., KUBOTA, M., TAKIMOTO, T., KITO, T., AKIYAMA, Y., KIRIYAMA, Y., AND MIKAWA, H.: Synergistic effect of methotrexate and 1- -D- arabinofuranosylcytosine on the generation of DNA strand breaks in a human promyelocytic leukemia cell line. Leuk. Res. 13: 151–156, 1989.

494. TANNOCK, I.: Response of aerobic and hypoxic cells in a solid tumor to adriamycin and cyclophosphamide and interaction of the drugs with radiation. Cancer Res. 42: 4921–4926, 1982.

495. TATTERSALL, M. H. N., HUTCHISON, R. M., GAYA, H., AND SPEIRS, A. D.: Empirical antibiotic therapy in febrile patients with neutropenia and malignant disease. Eur. J. Cancer 9: 417–423, 1973.

496. TAYLOR, S. H., SILKE, B., AND NELSON, G. I. C.: Principles of treatment of left ventricular failure. Eur. Heart J. (suppl. D) 3: 19–43, 1982.

497. TEICHER, B. A., GUNNER, L. J., AND ROACH, J. A.: Chemopotentiation of mitomycin C cytotoxicity *in vitro* by platinum complexes. Br. J. Cancer 52: 833–839, 1985.

498. TEICHER, B. A., HOLDEN, S. A., CUCCHI, C. A., CATHCART, K. W. S., KORBUT, T. T., FLATOW, J. L., AND FREI, E., III: Combination of N,N',N'' triethylenethiophosphoramide and cyclophosphamide *in vitro* and *in vivo*. Cancer Res. 48: 94–100, 1988.

499. TELLER, M. N., STOCK, C. C., BOWIE, M., CHOU, T-C., AND BUDINGER, J. M.: Therapy of 7,12-dimethylbenz(a)anthracene-induced rat mammary carcinomas with combinations of 5-fluorouracil and 2α-methyldihydrotestosterone propionate. Cancer Res. 42: 4408–4412, 1982.

500. THOMAS, D. C., AND WHITTEMORE, A. S.: Methods for testing interactions, with applications to occupational exposures, smoking, and lung cancer. Am. J. Indust. Med. 13: 131–147, 1988.

501. THOMAS, F. E., JR., LEONARD, J. M., AND ALFORD, R. H.: Sulfamethoxazole-trimethoprim-polymyxin therapy of serious multiply drug-resistant *Serratia* infections. Antibiot. Agents Chemother. 9: 201–207, 1976.

502. TIERNEY, P. C., KIM, Y. I., AND JOHNS, T. R.: Synergistic interaction of 4-aminopyridine with neostigmine at the neuromuscular junction. Eur. J. Pharmacol. 115: 241–247, 1985.

503. TRAUB, W. H., KOHL, K-H., SPOHR, M., AND BAUER, D.: Failure of polymyxin B nonapeptide to augment bactericidal acivities of novobiocin, rifampin, and defibrinated human blood against *Serratia marcescens*. Chemotherapy 34: 195–201, 1988.

504. TSAI, C-M., GAZDAR, A. F., VENZON, D. J., STEINBERG, S. M., DEDRICK, R. L., MULSHINE, J. L., AND KRAMER, B. S.: Lack of *in vitro* synergy between etoposide and *cis*-diamminedichloroplatinum(II). Cancer Res. 49: 2390–2397, 1989.

505. TURNER, M. E., JR.: Some classes of hit-theory models. Math. Biosci. 23: 219–235, 1975.

505a. TVERSKOY, M., BEN-SHLOMO, I., EZRY, J., FINGER, J., AND FLEYSHMAN, G.: Midazolam acts synergistically with methohexitone for induction of anaesthesia. Br. J. Anaesth. 63: 109–112, 1989.

506. TVERSKOY, M., FLEYSHMAN, G., BRADLEY, E. L., JR., AND KISSIN, I.: Midazolam-thiopental anesthetic interactions in patients. Anesth. Analg. 67: 342–345, 1988.

507. TVERSKOY, M., FLEYSHMAN, G., EZRY, J., BRADLEY, E. L., JR., AND KISSIN, I.: Midazolam-morphine sedative interactions in patients. Anesth. Analg. 68: 282–285, 1989.

508. TYRELL, R. M.: Radiation synergism and antagonism. Photochem. Photobiol. Rev. 3: 35–113, 1978.

509. UNKELBACH, H. D., AND PÖCH, G.: Comparison of independence and additivity in drug combinations. Arzneimittelforschung 38: 1–6, 1988.

510. UNKELBACH, H. D., AND WOLF, T.: Drug combinations—concepts and terminology. Arzneimittelforschung 34: 935–938, 1984.

511. UNKELBACH, H. D., AND WOLF, T.: Dose-response analysis of combination preparations. Stat. Med. 4: 77–85, 1985.

512. UBSINA, J. A., LAZARDI, K., AGUIRRE, T., PIRAS, M. M., AND PIRAS, R.: Antiproliferative synergism of the allylamine SF 86–327 and ketoconazole on epmastigotes and amastigotes of *Trypanosoma (Schistotrypanum) cruzi*. Antibiot. Agents Chemother. 32: 1237–1242, 1988.

513. VALERIOTE, F., AND LIN, H-S.: Synergistic interactions of anticancer agents: a cellular perspective. Cancer Chemother. Rep. 59: 895–900, 1975.

514. VELDSTRA, H.: Synergism and potentiation with special reference to the combination of structural analogues. Pharmacol. Rev. 8: 339–387, 1956.

515. VERRIOPOULOS, C., MORAITOU-APOSTOLOPOULOU, M., AND MILLOU, E.: Combined toxicity of four toxicants (Cu, Cr, oil, oil dispersant) to *Artemia salina*. Bull. Environ. Contam. Toxicol. 38: 483–490, 1987.

516. VERTUT-CROQUIN, A., BRAJTBURG, J., AND MEDOFF, G.: Two mechanisms of synergism when amphotericin B is used in combination with actinomycin D or 1-(2chloroethyl)-3-cyclohexyl1nitrosourea against the human promyelocyte leukemia cell line HL-60. Cancer Res. 46: 6154–6158, 1986.

517. VICENTINI, L. M., AND VILLAREAL, M. L.: Activation of Na^+/H^+ exchange in cultured fibroblasts: synergism and antagonism between phorbol ester, Ca^{2+} ionophore, and growth factors. Proc. Nat. Acad. Sci. 82: 8053- 8056, 1985.

518. VOGT, M. W., DURNO, A. G., CHOU, T-C., COLEMAN, L. A., PARADIS, T. J., SCHOOLEY, R. T., KAPLAN, J. C., AND HIRSCH, M. S.: Synergistic interaction of 2',3'-deoxycytidine and recombinant interferon-α-A on replication of human immunodeficiency virus type 1. J. Infect. Dis. 158: 378–385, 1988.

519. VON KROGH, M.: Colloidal chemistry and immunology. J. Infect. Dis. 19: 452–477, 1916.

520. WAAGE, A., AND ESPEVIK, T.: Interleukin 1 potentiates the lethal effect of tumor necrosis factor in mice. J. Exper. Med. 167: 1987–1992, 1988.

521. WADE, G. G., MANDEL, R., AND RYSER, H. J-P.: Marked synergism of dimethylnitrosamine carcinogenesis in rats exposed to cadmium. Cancer Res. 47: 6606–6613, 1987.

522. WADLER, S., GREEN, M. D., AND MUGGIA, F. M.: Synergistic activity of doxorubicin and the bisdioxopiperazine(+)-1,2bis(3,5-dioxopiperazinyl-1-yl) propane (ICRF 187) against the murine sarcoma S180 cell line. Cancer Res. 46: 1176–1181, 1986.

523. WAHLSTRÖM, G. AND NORBERG, A.: A comparative investigation in the rat of the anesthetic effects of the isomers of two barbiturates. Brain Res. 310: 261–267, 1984.

524. WALLIN, R. F., BLACKBURN, W. H., AND NAPOLI, M. D.: Pharmacologic interactions of albutoin with other anticonvulant drugs. J. Pharmacol. Exp. Ther. 174: 276–282, 1970.

525. WALTER, S. D., AND HOLFORD, T. R.: Additive, multiplicative and other models for disease risks. Am. J. Epidemiol. 108: 341–346, 1978.

526. WAMPLER, G. L., CARTER, W. H., JR., CAMPBELL, E. D., AND GOLDMAN, I. D.: Demonstration of a schedule-dependent therapeutic synergism utilizing the interacting drugs methotrexate and teniposide in L1210 leukemia. Cancer Treat. Rep. 71: 581–591, 1987.

527. WAMPLER, G. L., CARTER, W. H., JR., AND WILLIAMS, V. R.: Combination chemotherapy: arriving at optimal treatment levels by incorporating side effect constraints. Cancer Treat. Rep. **62**: 333–340, 1978.

528. WARE, J. A., SMITH, M., AND SALZMAN, E.W.: Synergism of platelet-aggregating agents. Role of elevation of cytoplasmic calcium. J. Clin. Invest. **80**: 267–271, 1987.

529. WATANABE, N., NIITSU, Y., UMENO., H., SONE, H., NEDA, H., YAMAUCHI, N., MAEDA, M., AND URUSHIZAKI, I.: Synergistic cytotoxic and antitumor effects of recombinant human tumor necrosis factor and hyperthermia. Cancer Res. **48**: 650–653, 1988.

530. WATSON, J. W., DRAKEN, J. M., AND STIMLER-GERARD, N. P.: Synergism between inflammatory mediators *in vivo*. Induction of airway hyperresponsiveness to C3a in the guinea pig. Am. Rev. Respir. Dis. **137**: 636–640, 1988.

531. WEBB, J. L.: Enzyme and Metabolic Inhibitors, Vol. 1, Academic Press, New York, 1963.

532. WEBB, R. B., BROWN, M. S., AND TYRRELL, R. M.: Synergism between 365- and 254-nm radiations for inactivation of *Escherichia coli*. Radiat. Res. **74**: 298–311, 1978.

533. WEBER, L. J., ANDERSON, P., MUSKA, C., YINGER, E. K., AND SHELTON, D.: Concentration and response addition of mixtures of toxicants using lethality, growth and organ system studies. *In* ASA/EPA Conferences on Interpretation of Environmental Data. 1. Current Assessment of Combined Toxicant Effects. May 5–6, 1986, pp. 1–35, USEPA, Washington, D.C., 1986.

534. WEED, D. L., SELMON, M., AND SINKS, T.: Links between categories of interaction. Am. J. Epidemiol. **127**: 17–27, 1988.

535. WEINBERG, J. B., AND LARRICK, J. W.: Receptor-mediated monocytoid differentiation of human promyelocytic cells by tumor necrosis factor: synergistic actions with interferon-γ and 1,25-dihydroxyvitamin D_3. Blood **70**: 994–1002, 1987.

536. WEINBERG, J. B., MISUKONIS, M. A., HOBBS, M. M., AND BOROWITZ, M. J.: Cooperative effects of gamma interferon and 1-alpha, 25-dihydroxyvitamin D_3 in inducing differentiation of human promyelocyte leukemia (HL-60) cells. Exp. Hematol. **14**: 138–142, 1986.

537. WEINSTEIN, R. J., YOUNG, L. S., AND HEWITT, W. L.: Comparison of methods for assessing in vitro antibiotic synergism against *Pseudomonas* and *Serratia*. J. Lab. Clin. Med. **86**: 853–862, 1975.

538. WELANDER, C. E., MORGAN, T. M., HOMESLEY, H. D., TROTTA, P. P., AND SPIEGEL, R. J.: Combined recombinant human interferon $alpha_2$ and cytotoxic agents studied in a clonogenic assay. Int. J. Cancer **35**: 721–729, 1985.

539. WERKHEISER, W. C.: Mathematical simulation in chemotherapy. Ann. N.Y. Acad. Sci. **186**: 343–358, 1971.

540. WERKHEISER, W. C., GRINDEY, G. B., MORAN, R. G., AND NICHOL, C. A.: Mathematical simulation of the interaction of drugs that inhibit deoxyribonucleic acid biosynthesis. Mol. Pharmacol. **9**: 320–329, 1973.

541. WESSINGER, W. D., AND EVANS, E. B.: Modeling multiple agent interactions in behavioral pharmacology. J. Am. Coll. Toxicol. **7**: 953–962, 1988.

542. WILLIAMS, T. J.: Prostaglandin E_2, prostaglandin I_2 and the vascular changes of inflammation. Br. J. Pharmacol. **65**: 517–524, 1979.

543. WILLIAMS, T. J., AND MORLEY, J.: Prostaglandins as potentiators of increased vascular permeability in inflammation. Nature **246**: 215–217, 1973.

544. WILLIAMS, T. J., AND MORLEY, J.: Role of prostaglandin-mediated vasodilatation in inflammation. Nature **270**: 530–532, 1977.

545. WILSON, J. D., CARTER, W. H., JR., CAMPBELL, E. D., KESSLER, F. K., AND CARCHMAN, R. A.: Application of response-surface methodology to detect interactions of genotoxic agents in cultured mammalian cells. J. Toxicol. Environ. Health **19**: 173–183, 1986.

546. WITTES, R. E., AND GOLDIN, A.: Unresolved issues in combination chemotherapy. Cancer Treat. Rep. **70**: 105–125, 1986.

547. WOLF, T., AND UNKELBACH, H. D.: The concept of additivity of drug combinations in the case of quantal response. Biometrics J. **4**: 453–461, 1985.

548. WOLFE, J. D., TASHKIN, D. P., CALVARESE, B., AND SIMMONS, M.: Bronchodilator effects of terbutaline and aminophylline alone and in combination in asthmatic patients. N Engl. J. Med. **298**: 363–367, 1978.

549. WOODWARD, D. F., NIEVES, A. L., WILLIAMS, L. S., GARY, R. K., JR., WASSERMAN, M. A., AND GLEASON, J. G.: Interactive effects of peptidoleukotrienes and histamine on microvascular permeability and their involvement in experimental cutaneous and conjunctival immediate hypersensitivity. Eur. J. Pharmacol. **164**: 323–333, 1989.

550. WOODWARD, J., LIMA, M., AND LEE, N. E.: The role of cellulase concentration in determining the degree of synergism in the hydrolysis of microcrystalline cellulose. Biochem. J. **255**: 895–899, 1988.

551. WOOLFOLK, C. A., AND STADTMAN, E. R.: Regulation of glutamine synthetase III. Cumulative feedback inhibition of glutamine synthetase from *Escherichia coli*. Arch. Biochem. Biophys. **118**: 736–755, 1967.

552. WORLD HEALTH ORGANIZATION STUDY GROUP: Chemotherapy of leprosy for control programmes. World Health Organization Technical Report Series 675, World Health Organization, Geneva, 1982.

553. WU, D-Z., ZHANG, Y-Q., KENG, P., SUTHERLAND, R. M., AND LASAGNA, L.: The interaction between bleomycin and radiation on cell survival and DNA damage in mammalian cell cultures. Int. J. Radiat. Oncol. Biol. Phys. **11**: 2125–2141, 1985.

554. YANG, R. S. H., AND GOEHL, T. J.: Toxicology studies of a chemical mixture of 25 groundwater contaminants: (I) chemistry development. Fund. Appl. Toxicol. In press.

555. YANG, R. S. H., AND RAUCKMAN, E. J.: Toxicological studies of chemical mixtures of environmental concern at the National Toxicology Program: Health effects of groundwater contaminants. Toxicology **47**: 15–34, 1987.

556. YEUNG, J. C., AND RUDY, T. A.: Multiplicative interaction between narcotic agonisms expressed at spinal and supraspinal sites of antinociceptive action as revealed by concurrent intrathecal and intracerebroventricular injections of morphine. J. Pharmacol. Exp. Ther. **215**: 633–642, 1980.

557. YONETANI, T., AND THEORELL, H.: Studies on liver alcohol dehydrogenase complexes. III. Multiple inhibition kinetics in the presence of two competitive inhibitors. Arch. Biochem. Biophys. **106**: 243–251, 1964.

558. YOUNG, L. S.: *Mycobacterium avium* complex infection. J. Infect. Dis. **157**: 863–867,1988.

559. YU, V. L., FEIEGIE, T. P., YEE, R. B., PASCULLE, A. W., AND TAYLOR, F. H.: Synergistic interaction in vitro with use of three antibiotics simultaneously against *Pseudomonas maltophilia*. J. Infect. Dis. **142**: 602–607, 1980.

560. YU, V. L., ZURAVLEFF, J. J., BORNHOLM, J., AND ARCHER, G.: *In vitro* synergy testing of triple antibiotic combinations against *Staphylococcus epidermidis*. J. Antimicrob. Chemother. **14**: 359–366, 1984.

561. ZINNER, S. H., KLASTERSKY, J., GAYA, H., BERNARD, C., RYFF, J. C., AND THE EORTC ANTIMICROBIAL PROJECT GROUP: In vitro and in vivo studies of three antibiotic combinations against gram-negative bacteria and *Staphylococcus aureus*. Antibiot. Agents Chemother. **20**: 463–469, 1981.

562. ZISKIND, A. A., GOLD, H. K., YASUDA, T., KANKE, M., GUERRERO, J. L., FALLON, J. T., SAITO, T., AND COLLEN, D.: Synergistic combinations of recombinant human tissue-type plasminogen activator and human single-chain urokinase-type plasminogen activator. Effect on thrombolysis and reocclusion in a canine coronary artery thrombosis model with high-grade stenosis. Circulation **79**: 393–399, 1989.

563. ZUCKERMAN, S. H., AND SURPRENANT, Y. M.: Induction of endothelial cell/macrophage procoagulant activity: synergistic stimulation by gamma interferon and granulocyte-macrophage colony stimulating factor. Thromb. Haemostas. **61**: 178–182, 1989.

564. ZUCKERMAN, S. H., SURPRENANT, Y. M., AND TANG, J.: Synergistic effect of granulocyte-macrophage colony-stimulating factor and 1,25-dihydroxyvitamin D_3 on the differentiation of the human monocyte cell line U937. Blood **71**: 619–624, 1988.