Sequential Blockage as a Theoretical Basis for Drug Synergism

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Kinetics analysis of a linear model enzyme system shows that, in theory, the combined effect of two inhibitors acting by sequential blockage is *necessarily* synergistic. The rate equations for the system describe a theoretical dose-effect surface for the drug pair which results in a formal definition of synergism that correlates well with experimental observation over the concentration range for which the effect is demonstrable. This definition provides a rationale for the isobole technique for demonstrating synergism experimentally and a means for defining and calculating the "amount" of synergism shown by two drugs.

The term synergism[1] has been applied by pharmacologists[2] and chemotherapists[3-6] to instances in which two or more inhibitors of a biological response are more effective when acting together than would be expected[7] from additivity of their individual effects.[8]

Synergism is often encountered[9] and is always of interest for the possibility that host toxicity may be only additive,[10] or that the emergence of drug resistance may be delayed by the combined use of two or more inhibitors with different modes of action.[11] The effect is unmistakable[12] when drugs A and B, which produce a certain sub-maximal effect in concentrations a and b alone, produce the same effect when combined in concentrations xa and yb, with $(x + y) <<< 1$. Results of this type seem to be in some way multiplicative, instead of additive, functions of inhibitor concentrations, but they apparently have never been accounted for in theoretical terms.[13]

The plausibility of sequential blockage as a mechanism for synergism is widely accepted,[13,44] but on purely intuitive grounds and not without dissent.[14] It is the purpose of the present work to show that synergism in this sense is not only *possible* but is, indeed, a *necessary* consequence of a sequentially blocked mechanism. This will be done by deriving from the kinetics of a model reaction an expression for the net effect of two inhibitors that act at *different* points in a linear sequence of enzymatic reactions. The resulting expression leads to a formal definition of synergism that relates individual dose-effect curves to a dose-effect, or boloform,[8] surface for joint inhibition. This expression contains a small number of constants, each of which is a defined reaction parameter. Synergism, as defined by this equation, is a *necessary* result of sequential blockage in the sense that the net effect of the inhibitors is a multiplicative function of their concentrations for *all possible values* of the constants and variables that the defining equation contains.

The model to be adopted is based, in part, upon these assumptions: (1) the intensity (R_t) of the biological response in question, whether inhibited or not, is limited at a given time by (and *only* by) the instantaneous rate, dS_3/dt, at which some metabolite, S_3, is being produced at the same time from precursors, S_1 and S_2, by the mechanism, $S_1 \rightarrow S_2 \rightarrow S_3$; (2) the effect ($E_t$) of inhibitors of the response is to depress the rate of formation of S_3; (3) each inhibitor acts by competing with a different substrate for active sites on the enzyme, E_1 or E_2, with which the substrate must combine to react.

These assumptions will be introduced later in more explicit mathematical form; they are more general than those upon which the Michaelis–Menten theory is based, so in this sense the present results are more general than a Michaelis–Menten treatment of this specific

(1) Observations of synergism and use of the isobole method for describing them are at least 90 years old. T. R. Fraser, *Brit. Med. J.*, **2**, 457, 485 (1872), did not use the term but observed synergism, as well as antagonism, between atropine and physostigmine, depending on relative doses. Since Fraser, the term has had a controversial career, as emphasized by a recent exchange of Letters to the Editor, Various Authors, *The New Scientist*, **14**, 481, 600, 662 (1962). The term even has a metaphysical connotation; *ibid.*, **15**, 45 (1962).

(2) G. Chen, *Arch. Intern. Pharmacodyn.*, **111**, 322 (1957).

(3) G. A. H. Buttle, *Proc. Roy. Soc. Med.*, **49**, 873 (1956).

(4) E. Jawetz and J. B. Gunnison, *Pharmacol. Revs.*, **5**, 175 (1953).

(5) E. Jawetz, J. B. Gunnison and V. R. Coleman, *J. Gen. Microbiol.*, **10**, 191 (1954).

(6) Anon., *Chem. Eng. News*, **32**, 4473 (1954).

(7) One person will "expect" more than another, so this "definition" is operationally worthless. A. J. Zwart Voorspuij and C. A. G. Nass, *Arch. Intern. Pharmacodyn.*, **109**, 211 (1957), and S. Loewe, *Arzneimittel-Forsch.*, **3**, 285 (1953), have in fact shown how it can lead to the absurd conclusion that a drug can be synergistic with itself. As Loewe contends, the decisive test for synergism lies in treatment of the data by the boloform method. This method has been applied to double inhibition by G. H. Hitchings, *Am. J. Clin. Nutrition*, **3**, 321 (1955), by S. B. Kendall, *Proc. Roy. Soc. Med.*, **49**, 874 (1956), and by many others. Chen, ref. 2, and A. J. Zwart Voorspuij and L. H. Bokma, *Ann. inst. Pasteur*, **95**, 404 (1958), have used 4-dimensional boloforms to describe the joint effects of 3 inhibitors.

(8) Loewe (see ref. 7) emphasizes that a definition of synergism based on non-additivity *vs.* additivity of *effects* is meaningless since effects are *never* with certainty additive. He discusses in detail the question of "what is properly additive to what." See also ref. 21.

(9) The literature on synergism has been reviewed by H. Veldstra, *Pharmacol. Revs.*, **8**, 339 (1956). A well documented case results from the action of sulfadiazine (I_1) and pyrimethamine (I_2) on a variety of organisms, both *in vitro* and *in vivo*, presumably by blockage of the essential sequence

$$p\text{-Aminobenzoic acid} \xrightarrow{I_1} \text{Folic acid} \xrightarrow{I_2} \text{Folinic acid}$$

See L. G. Goodwin, *Proc. Roy. Soc. Med.*, **49**, 871 (1956); S. B. Kendall, see ref. 7; L. G. Goodwin and J. M. Rollo in "The Biochemistry and Physiology of Protozoa," S. H. Hutner and A. Lwoff, eds., Academic Press, New York, N. Y., 1955, Vol. 2, pp. 245–246; L. P. Joyner and S. B. Kendall, *Nature*, **176**, 975 (1955); G. H. Hitchings, see ref. 7.

(10) As was shown by J. Greenberg, B. L. Boyd, and E. S. Josephson, *J. Pharmacol. Exptl. Therap.* **94**, 60 (1948), for experimental treatment of *Plasmodium gallinaceum* infections with sulfadiazine–chloroguanide combinations.

(11) D. A. Mitchison, *Brit. Med. Bull.*, **18**, 77 (1962).

(12) See, for example, M. W. Fisher and L. Doub, *Biochem. Pharmacol.*, **3**, 10 (1959). M. W. Fisher, *Antibiot. Chemotherapy*, **7**, 315 (1957), also has shown that a concerted inhibitory effect, consisting of antimetabolic and immunogenic components, can achieve the same net result. B. A. Waisbren, *ibid.*, **7**, 322 (1957), has reported the clinical effectiveness of this technique.

(13) A. Albert, *Proc. Roy. Soc. Med.*, **49**, 881 (1956), comments briefly on the "arithmetic" of synergism but does not show how inhibitor concentrations are involved.

(14) See, for example, R. Knox, *ibid.*, **49**, 879 (1956).

(15) M. Dixon and E. C. Webb, "Enzymes," Academic Press, Inc., New York, N. Y., 1958, Chapt. XII, discuss substrate-linked multi-enzyme systems in detail.

(16) The interdependence of bacterial growth rates and enzyme-substrate kinetics is treated exhaustively by C. N. Hinshelwood, "The Chemical Kinetics of the Bacterial Cell," Oxford University Press, London, 1946. See also M. Harris and G. A. Morrison, *Nature*, **191**, 1276 (1961).

problem would allow. The latter theory[17,18] has dealt with the effect of a single inhibitor upon a single enzymatic step but has not been developed in a direction that fits the present purpose. The non-biological literature[19] on consecutive reactions also fails to show how inhibitor concentrations combine to affect net reaction rates.

The reaction model assumed here is, of course, an over-simplification[20]; its usefulness is nevertheless shown by the identity of the resulting rate equations for single inhibition with certain empirical expressions that have been applied successfully to a variety of singly-inhibited biological responses. The equations to be developed therefore suggest a deductive basis for these empirical expressions and permit for the first time a presumptive identification of the arbitrary constants that they contain.

The equation for joint inhibition defines a theoretical dose-effect, or boloform, surface; the validity of this equation as a definition of synergism is supported by the similarity of constant-effect profiles of this surface to published isoboles[8,41,44] whose shape is commonly accepted as distinctive of synergism. These and other points of agreement with published work will be discussed later in detail; they suggest that, despite the highly idealized nature of the model, sequential blockage is *necessarily* synergistic over a concentration range that is experimentally meaningful; they also imply that additive[21] and antagonistic[22] effects of jointly administered drugs should depend upon mechanisms that differ in some fundamental way from one that results in synergism.

The present results indicate that synergism is as much a feature of the enzyme system as it is a feature of the inhibitors; they do not deny, however, that types of synergism entirely unrelated to sequential blockage may also exist.[14,45]

Derivation of Rate Equations

Symbols and Abbreviations.—The conventions and assumptions of chemical, bacterial and enzyme kinetics to be used involve the following terminology.

s_1^0, s_2^0, s_3^0, i_1^0, i_2^0—concentrations of free substrates, S_1, S_2, and S_3 and free inhibitors, I_1 and I_2, at time, $t = 0$. i_1^0 and i_2^0 are synonymous with concentrations of drugs added at the beginning of an experiment.

s_1, s_2, s_3, i_1, i_2—instantaneous concentrations of free S_1, S_2, S_3, I_1, and I_2 at time, t.

e_1, e_2—instantaneous concentrations of free enzymatic sites, E_1 and E_2, at time, t.

t—time, dating from some arbitrary beginning, $t = 0$.

k_1, k_2, k_3, k_4—specific rate constants for reactions in the models described below.

$\alpha = k_3/k_4$

R_t—response of the system as limited by the instantaneous rate of appearance of S_3 at time, t, with initial inhibitor concentrations, i_1^0 and i_2^0, either alone or combined.

E_t—drug effect at time, t.

f—functional notation, as in $E_t = f(i_1^0, i_2^0)$. Use in different connotations does not imply that f is of the same form in all cases.

F—the function corresponding to f after integration.

C—integration constant; non-committal in same sense as f.

ln—logarithm to base e.

e—base of the natural logarithm system.

n_t—instantaneous concentration of bacterial cells in a logarithmically growing culture at time, t.

k'—bacterial growth rate constant during the logarithmic growth phase.

P—proportionality constant relating instantaneous bacterial growth rate to instantaneous rate of appearance of S_3.

A_t—optical absorbancy of a bacterial culture containing a concentration of cells, n_t, at time, t.

q—proportionality constant relating A_t and n_t such that $A_t = qn_t$.

Σ—integrated "amount" of synergism shown by the two inhibitors relative to a specified E_t end-point.

Kinetics Definitions

First Order Case

(a) $-ds_1/dt = k_1 s_1$

(b) $-di_1/dt = k_4 i_1$

(c) $-ds_2/dt = k_2 s_2 = ds_3/dt$

(d) $-di_2/dt = k_4 i_2$

(e) $R_t = ds_3/dt = k_2 s_2$. This is a mathematical restatement of Assumption 1 (see Introduction).

(f) $E_t \propto 1/R_t$, whence $dE_t/E_t = -dR_t/R_t$. This is a mathematical re-statement of assumption 2 (see introduction).

(g) $i_1 = i_1^0 e^{-k_4 t}$ (by integration of definition b)

(h) $i_2 = i_2^0 e^{-k_4 t}$ (by integration of definition d)

(i) If the system is closed, then, by material balance

$$s_1 + s_2 + s_3 = s_1^0 + s_2^0 + s_3^0$$

(j) For the specific case of bacterial growth

$dn_t/dt = k' n_t = P(R_t) = k' A_t/q$ during the logarithmic growth phase.

Second Order Case

(k) $-ds_2/dt = k_2 e_2 s_2 = ds_3/dt = R_t$

(l) $-ds_1/dt = k_1 e_1 s_1$

(m) $-di_1/dt = k_3 e_1 i_1$

(n) $-di_2/dt = k_3 e_2 i_2$

Mathematical Definitions

The usual notation of differential and integral calculus will be used, including the two theorems

(A) If $x = f(y,z)$, then

$$dx = (\partial x/\partial y)_z dy + (\partial x/\partial z)_y dz$$

even when y and z are interdependent.[23]

(B) If $w = f(x)$, $x = f(y)$, and $y = f(z)$ then[24]

$$\frac{dw}{dz} = \frac{dw}{dx}\,\frac{dx}{dy}\,\frac{dy}{dz}$$

Summary of Procedure.—Expressions for the functions

$$dR_t = f_1(di_1^0)$$

(17) Summarized in H. G. Bray and K. White, "Kinetics and Thermodynamics in Biochemistry," Academic Press Inc., New York, N. Y., 1957, Chap. 7.

(18) J. Z. Hearon, *Bull. Math. Biophys.*, **11**, 29, 83 (1949); **15**, 121 (1953), has generalized the Michaelis–Menten theory to include a connected sequence of enzymatic reactions, but has not shown the consequences of multiple inhibition. See also J. Z. Hearon, *Physiol. Rev.*, **32**, 499 (1952), for a general discussion.

(19) See, for example, J. W. Mellor, "Higher Mathematics for Students of Chemistry and Physics," Dover Publications, New York, N. Y., 1946, pp. 443–440; A. A. Frost and R. G. Pearson, "Kinetics and Mechanisms; a Study of Homogeneous Chemical Reactions," John Wiley and Sons, Inc., New York, N. Y., 1953, Chapt. 8.

(20) But necessarily so, for only the simplest mechanisms are amenable to complete kinetics analysis. In fact, a simple analytical solution for the transient-state kinetics of the Michaelis–Menten mechanism is not possible. B. Chance, *J. Biol. Chem.*, **151**, 553 (1953), obtained particular solutions by use of a differential analyzer. These were confirmed by C.-C. Yang, *Arch. Biochem. Biophys.*, **51**, 419 (1953), using the reversion method. G. B. Kistiakowsky and P. C. Mangeldorf, Jr., *J. Am Chem. Soc.*, **78**, 2964 (1956), achieved a close approximation by a power series expansion of the Michaelis–Menten rate equation.

(21) A rate equation for the additive effects of two inhibitors acting on the *same* enzymatic step has been deduced by F. H. Johnson, H. Eyring, and M. J. Polissar, "The Kinetic Basis of Molecular Biology," John Wiley and Sons, New York, N. Y., 1954, pp. 469–470.

(22) J. H. Gaddum, *J. Physiol.*, **89**, 7P (1937).

(23) V. H. Wells, "Elementary Calculus," D. Van Nostrand Co., Inc., New York, N. Y., 1941, p. 320.

(24) G. J. Kynch, "Mathematics for the Chemist," Academic Press Inc., New York, N. Y., 1955, p. 14.

$$dR_t = f_2(di_2^0)$$

will first be derived. These will be combined by theorem A to give an expression for

$$dR_t = f_{1,2}(di_1^0, di_2^0)$$

Because of the logarithmic nature of f_1 and f_2 and the relationship between R_t and E_t, the differential equation that results will be shown to have a solution of the form

$$E_t = F_{1,2}(i_1^0 \times i_2^0)$$

E_t is, thus, a function of the *product* of i_1^0 and i_2^0, proving that synergism is a *necessary* property of the model assumed. E_t, as a function of i_1^0 and i_2^0, is the equation for a boloform surface.[8] An isobole is a constant-effect profile of a boloform surface[7]; thus $dE_t = 0$, leaving an expression for $i_1^0 = F(i_2^0)$ as the equation for a theoretical isobole. The slope and direction-of-curvature of this isobole denote synergism for all possible values of the constants and variables.

Discussion of the Reaction Model.—Proof of the case for synergism requires an expression relating R_t to i_1^0 and i_2^0. The rate expression can contain i_1^0 and i_2^0 as variables only if $i_1 \sim e_1$ and $i_2 \sim e_2$ or if $e_1 >>> i_1$ and $e_2 >>> i_2$. Since competition between inhibitors and substrates for the enzyme surfaces is required for inhibition, $s_1 \sim i_1$ and $s_2 \sim i_2$ are also realistic conditions. The only models, therefore, that will permit a proof of the desired type are:

(1) 1st-order in s_1, s_2, i_1, i_2 and zero-order in e_1 and e_2; *i.e.*, $e_1 >>> s_1$ and i_1; $e_2 >>> s_2$ and i_2. A proof based on this model will be given in detail.

(2) 2nd-order in which $s_1 \sim i_1 \sim e_1$ and $s_2 \sim i_2 \sim e_2$. This procedure gives a more general but less useful result than the above. The differential equations that result cannot be integrated, but they reduce to the 1st-order set when 1st-order conditions are imposed. The 2nd-order case may be safely ignored for the present purpose, for reasons to be given later.

The arguments to follow do not require the assumption of an intimately detailed mechanism, but the 1st-order case may be depicted in highly schematic form as shown:

(1) Inhibition by I_1 alone

$$\left[\begin{array}{l} I_1 \xrightarrow[(E_1)]{k_x} (E_1)I_1 \\ S_1 \xrightarrow[(E_1)]{k_1} S_2 \end{array}\right] \xrightarrow[(E_2)]{k_2} S_3$$

(2) Inhibition by I_2 alone

$$S_1 \xrightarrow[(E_1)]{k_1} \left[\begin{array}{l} S_2 \xrightarrow[(E_2)]{k_2} S_3 \\ I_2 \xrightarrow[(E_2)]{k_y} (E_2)I_2 \end{array}\right]$$

(3) Inhibition by I_1 and I_2 simultaneously

$$\left[\begin{array}{l} I_1 \xrightarrow[(E_1)]{k_x} (E_1)I_1 \\ S_1 \xrightarrow[(E_1)]{k_1} \left[\begin{array}{l} S_2 \xrightarrow[(E_2)]{k_2} S_3 \\ I_2 \xrightarrow[(E_2)]{k_y} (E_2)I_2 \end{array}\right] \end{array}\right]$$

The brackets enclose the species that react competitively. The symbols (E_1) and (E_2) have no mathematical meaning but are included as reminders that enzyme-1 and enzyme-2 are involved in the usual catalytic sense. In the 2nd-order model the concentrations e_1 and e_2 of these enzymes must, however, be treated as variables.

All reactions are assumed to be irreversible because of the mass-action effect of $e >>> (i \sim s)$. Steady-state conditions are not deliberately imposed, but the net result, as will be shown later, is equivalent to having done so.

The condition for competitive inhibition (Assumption 3, see Introduction) will appear in the following development as negative signs for operators of the type $-ds/di$, and for the functions that they represent. This condition is justified by the following argument. Competition prevails as long as both S and I are present, so *any* change in s occurs in the face of competition from I, and conversely. Consider two hypothetical experiments that differ only in the infinitesimal extent to which competition has occurred after a given time interval; the relationship existing between the variables at the stated time is,

$$ds = kd(i^0 - i)$$

i.e., the increased extent, ds, to which S has *not* reacted with E is due to more efficient competition by I, as measured by the increased extent, $d(i^0 - i)$, to which I *has* reacted with E within the same time interval. This relationship carries a linear proportionality constant, k, since competition occurs on a one-for-one basis. Performing the indicated differentiation gives

$$-ds/di = k$$

The precise form of the function corresponding to the operator $-ds/di$ will appear later, but the above argument shows that it can be interpreted as a condition for competition and that, as such, it is always negative. The negative sign will hereafter be affixed to ds/di as a reminder that the function that it represents must likewise be negative.

This argument acknowledges only those changes in s and i that are due to competition proper; other contributions to the net changes in these quantities will be incorporated, as needed, in the form of the appropriate operators. In this sense, $-ds/di$ is actually a partial differential coefficient in which all influences upon s and i that do not result from competition are considered constant. As will be shown later, this mathematical interpretation of a metabolic block leads to variants of Huxley's[25] well-known allometric equation, $dy/dx = ky/x$, which has been applied to competitive biological interactions of widely variable types.[26]

Derivation for First-order Model with Inhibition by I_1 Alone.—From definition (e)

$$R_t = f(s_2) \quad (1)$$

From definition (i), with s_1^0, s_2^0, s_3^0 and s_3 constant

$$s_2 = f(s_1) \quad (2)$$

From the condition for competition between S_1 and I_1 for E_1

$$s_1 = f(i_1) \quad (3)$$

From definition (g), when t is constant

$$i_1 = f(i_1^0) \quad (4)$$

(25) J. S. Huxley, "Problems of Relative Growth," Methuen and Co., Ltd., London, 1932, p. 7.

(26) H. G. Bray and K. White, ref. 17, pp. 332-333.

So, from theorem B

$$\frac{dR_t}{di_1^0} = \frac{dR_t}{ds_2} \times \frac{ds_2}{ds_1}\left(-\frac{ds_1}{di_1}\right)\frac{di_1}{di_1^0} \quad (5)$$

in which the negative term is the condition for competition between I_1 and S_1 for E_1.

The operations indicated in eq. (5) are performed on definitions (a), (b), (c), (e) and (g). Differentiation of definition (e) gives

$$dR_t/ds_2 = k_2 \quad (6)$$

Definition (c) divided by definition (a) gives

$$\frac{ds_2}{ds_1} = \frac{k_2 s_2}{k_1 s_1} \quad (7)$$

Definition (a) divided by definition (b) gives

$$-\frac{ds_1}{di_1} = -\frac{k_1 s_1}{k_x i_1} \quad (8)$$

Differentiation of definition (g) with t constant gives

$$di_1/di_1^0 = e^{-k_x t} \quad (9)$$

Combining eq. (6–9) in the manner indicated by eq. (5) gives

$$\frac{dR_t}{di_1^0} = k_2\left(\frac{k_2 s_2}{k_1 s_1}\right)\left(-\frac{k_1 s_1}{k_x i_1}\right)e^{-k_x t} \quad (10)$$

Substituting definition (g) for i_1 in eq. (10) and cancelling identical terms gives

$$\frac{dR_t}{di_1^0} = -\frac{k_2(k_2 s_2)}{k_x i_1^0} \quad (11)$$

Disappearance of the t term still leaves time as a hidden variable in all subsequent equations; thus R_t will always refer to response, and E_t to effect, after a constant time interval dating from introduction of the inhibitor(s) to the system.

By definition (e), $k_2 s_2 = R_t$, so eq. (11) becomes, after rearranging

$$dR_t = -\frac{k_2}{k_x} \times \frac{R_t}{i_1^0} di_1^0 \quad (12)$$

Integration of eq. (12) gives

$$-\ln R_t = \frac{k_2}{k_x} \ln i_1^0 + \ln C \quad (13)$$

or, by definition (f)

$$\ln E_t = \frac{k_2}{k_x} \ln i_1^0 + \ln C \quad (14)$$

Derivation for First-order Model with Inhibition by I_2 Alone.—The derivation follows from definitions (c), (d), (e), and (h), theorem B, and the condition for competition by a method similar to the above. Thus

$$R_t = f(s_2) \quad (15)$$

$$s_2 = f(i_2) \quad (16)$$

and, at constant t

$$i_2 = f(i_2^0) \quad (17)$$

So

$$\frac{dR_t}{di_2^0} = \frac{dR_t}{ds_2}\left(-\frac{ds_2}{di_2}\right)\frac{di_2}{di_2^0} \quad (18)$$

As before, the negative term is the condition for competition between S_2 and I_2 for E_2.

The terms in eq. (18) are obtained from definitions (c), (c) and (d), and (h) respectively. The result, for constant t, is

$$\frac{dR_t}{di_2^0} = k_2\left(-\frac{k_2 s_2}{k_y i_2}\right)e^{-k_y t} \quad (19)$$

Substituting definition (h) for i_2 and definition (c) for $k_2 s_2$, cancelling identical terms and rearranging gives

$$dR_t = -\frac{k_2}{k_y} \times \frac{R_t}{i_2^0} di_2^0 \quad (20)$$

Integration of eq. (20) gives

$$-\ln R_t = \frac{k_2}{k_y} \ln i_2^0 + \ln C \quad (21)$$

or, by definition (f)

$$\ln E_t = \frac{k_2}{k_y} \ln i_2^0 + \ln C \quad (22)$$

Derivation for First-order Model Inhibited by I_1 and I_2 Combined.—With both inhibitors present

$$R_t = f(i_1^0, i_2^0) \quad (23)$$

so, by theorem A

$$dR_t = \left(\frac{\partial R_t}{\partial i_1^0}\right)_{i_2^0} di_1^0 + \left(\frac{\partial R_t}{\partial i_2^0}\right)_{i_1^0} di_2^0 \quad (24)$$

But the partial differential coefficients on the right side of eq. (24) are given by eq. (12) and (20) so eq. (24) becomes

$$dR_t = -\frac{k_2}{k_x}\frac{R_t}{i_1^0} di_1^0 - \frac{k_2}{k_y}\frac{R_t}{i_2^0} di_2^0 \quad (25)$$

Rearranging and integrating eq. (25) gives

$$-\ln R_t = \frac{k_2}{k_x} \ln i_1^0 + \frac{k_2}{k_y} \ln i_2^0 + \ln C \quad (26)$$

or, by definition (f)

$$\ln E_t = \frac{k_2}{k_x} \ln i_1^0 + \frac{k_2}{k_y} \ln i_2^0 + \ln C \quad (27)$$

The locus of eq. (27) is the desired theoretical dose-effect surface for the drug pair.

Although the addition required by eq. (24) would seem to imply additive effects for I_1 and I_2, the net result is multiplicative—and therefore synergistic—because of the presence of the logarithmic factors, $di_1^0/i_1^0 = d(\ln i_1^0)$ and $di_2^0/i_2^0 = d(\ln i_2^0)$, as seen in eq. (25). It is this feature that leads to eq. (27) and its associated dose-effect surface as the first recorded indication of the *obligatory* nature of synergism. This point will be explored later in more detail.

Derivations for the Second-order Model.—The procedure (See Appendix) is analogous to that for the 1st-order case except that e_1 and e_2 must be treated as variables and freer use of partial differentials must therefore be made in handling the appropriate definitions, (f), (i), (k)–(n).

The equations that result are, for single inhibition

$$\frac{dE_t}{E_t} = \frac{k_2}{k_x}\left(\frac{e_2 + s_2}{e_1}\right)\frac{di_1^0}{i_1^0} \quad (28)$$

and

$$\frac{dE_t}{E_t} = \frac{k_2}{k_y}\left(\frac{e_2 + s_2}{e_2}\right)\frac{di_2^0}{i_2^0} \quad (29)$$

and, for double inhibition

$$\frac{dE_t}{E_t} = \frac{k_2}{k_x}\left(\frac{e_2 + s_2}{e_1}\right)\frac{di_1^0}{i_1^0} + \frac{k_2}{k_y}\left(\frac{e_2 + s_2}{e_2}\right)\frac{di_2^0}{i_2^0} \quad (30)$$

These equations cannot be integrated as they stand, but they are comparable in form to eq. (14), (22), and (27)

for the 1st-order cases. The validity of eq. (28), (29), and (30) relative to that of the corresponding 1st-order equations is seen by applying the 1st-order condition, $e_1 \sim e_2 >>> s_2$; this leads to $(e_2 + s_2)/e_1 \sim 1$ and $(e_2 + s_2)/e_2 \sim 1$. Integration is now possible, with results identical with eq. (14), (22), and (27) for the 1st-order case.

All subsequent discussion will be simplified, but with no loss in generality, by minimizing the 2nd-order case. This will emphasize the early, exponentially rising part of the composite dose-effect surface, Fig. 1, described by the exponential form of eq. (27). This emphasis is justified by the experimental need for working at inhibitor concentrations that produce sub-maximal effects in order to demonstrate synergism at all.

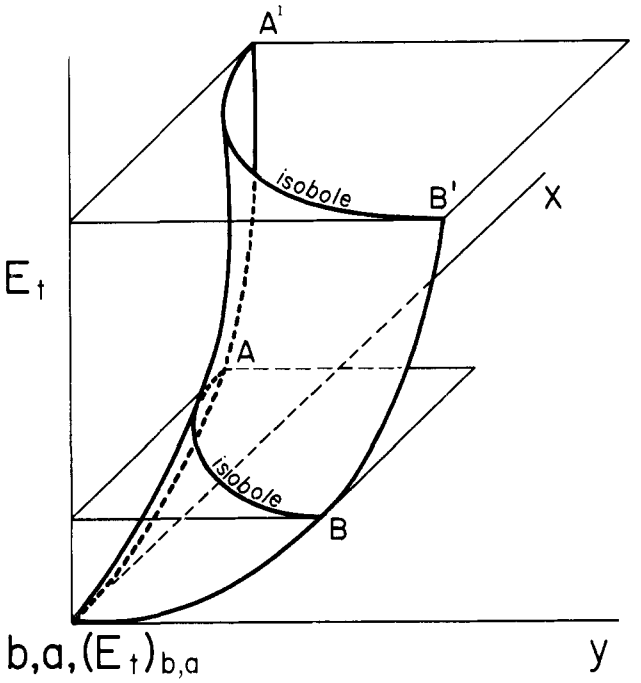

Fig. 1.—Part of the locus of the exponential form of eq. (27), developed for the 1st-order region in which $e_1 >>> (s_1 \sim i_1^0)$, $e_2 >>> (s_2 \sim i_2^0)$, $k_2 > k_x$ and $k_2 > k_y$. The qualitative identity of all analytic properties of this surface to those of an experimental boloform surface[8] is evidence for the validity of eq. (27) as a definition of synergism. The symmetry of the surface relative to the $i_1^0 = i_2^0$ plane depends on the magnitude of k_x/k_y. Definition of the individual dose-effect curves (AA' and BB') and the isobole intercepts (A, B, A', B') requires translation of eq. (27) and (34) to the origin shown here. This translation and the relevance of points A, a, B, b and coördinates x and y to an experimental isobole are discussed in connection with Fig. 3. The transition of an individual dose-effect curve, for example BB', to the familiar sigmoid form at higher i_2^0 values is explainable as a continuous transition to 2nd-order kinetics in the neighborhood of the inflection point (not shown) and to kinetics 0-order in inhibitor as the plateau (also not shown) is reached. In the plateau region, E_t is, in theory and in fact, independent of further increases in inhibitor concentrations. The notion of synergism is experimentally meaningless in the plateau region, so the inability of the model to account for it when $i_1^0 >>> e_1$ and $i_2^0 >>> e_2$ is expected. The E_t-coördinate of the origin, $(E_t)_{b,a}$, is given by eq. (43) and the individual dose-effect curves are defined by eq. (42) when $x = 0$ or $y = 0$.

Synergism is, of course, demonstrable for inhibitor concentrations in the 2nd-order region of the dose-effect surface, described by eq. (30); no inflections or discontinuities are discernible, however, in the approach of $(e_2 + s_2)/e_1$ and $(e_2 + s_2)/e_2$ to unity, which reduces eq. (30) to eq. (27), so conclusions as to the nature of synergism in the 2nd-order region should be qualitatively the same as those to follow for the more manageable 1st-order region, depicted in Fig. 1.[26a]

Other points of qualitative equivalence of the 1st- and 2nd-order cases will be emphasized as they appear. The fuller relevance of Fig. 1 to synergism will also appear later.

The steady-state assumption, $ds_2/dt = 0$, has not been deliberately imposed. This assumption is most often made for the mathematical simplicity to which it leads, but it also has a very realistic basis when applied to successive enzymatic reactions.[15] The results for the 1st-order model would be unchanged by this assumption since s_2 does not appear in the final equations. The 2nd-order model is reduced to 1st-order by assuming s_2 small relative to e_1 and e_2; this is equivalent, in its net effect upon E_t, to assuming s_2 constant, which is the steady-state condition.

The following discussion will be based upon results for the model inhibited at adjacent points

$$S_1 \xrightarrow{I_1} S_2 \xrightarrow{I_2} S_3$$

but the more general nature[18] of these results can be shown by assuming that the points of inhibition are *not* adjacent. If, for example

$$S_1 \underset{I_1}{\overset{k_1}{\rightarrow}} S_2 \xrightarrow{k_2} S_3 \underset{I_2}{\overset{k_3}{\rightarrow}} S_4 \xrightarrow{k_4} S_5$$

then by analogy with eq. (5)

$$\frac{dR_t}{di_1^0} = \frac{dR_t}{ds_4} \times \frac{ds_4}{ds_3} \times \frac{ds_3}{ds_2} \times \frac{ds_2}{ds_1}\left(-\frac{ds_1}{di_1}\right)\frac{di_1}{di_1^0}$$

or

$$\frac{dR_t}{di_1^0} = k_4\frac{k_1 s_4}{k_3 s_3} \times \frac{k_3 s_3}{k_2 s_2} \times \frac{k_2 s_2}{k_1 s_1}\left(-\frac{k_1 s_1}{k_x i_1}\right)e^{-k_x t}$$

This, after the operations of eq. (10), (11) and (12) gives, for inhibition by I_1 alone

$$dR_t = -\frac{k_4}{k_x} \times \frac{R_t}{i_1^0}\, di_1^0$$

a form identical with eq. (12). Results analogous to eq. (20) and (25) are also obtained for inhibition of the above sequence by I_2 and $I_1 + I_2$, so the following discussion applies to an unbranched sequence of *any* length inhibited at *any* two *different* points.

Discussion of Results

The dose-effect curve for a single inhibitor can often be linearized over an important part of its range by plotting on log–log axes. This technique has given linear plots for effects as diverse as: urea denaturation of tobacco mosaic virus[27]; carbamate inhibition of luminescence of *Photobacterium phosphoreum*[28]; phenobarbital inhibition of respiration of rat brain slices[29];

(26a) A referee has correctly observed that competition would be relatively inefficient in the 1st-order case; this is equivalent, of course, to recognizing that a small amount of drug has a small effect—a larger amount, a larger effect, *etc.*, until the plateau of the dose-effect curve is reached. The continuous collapse of the 2nd- to the 1st-order equations as 1st-order conditions are approached shows, however, that the condition for competition is applicable *in principle* over the entire concentration range, however small the extent to which competition might *actually* prevail in the extreme 1st-order case.

(27) M. A. Lauffer, *J. Am. Chem. Soc.*, **65**, 1793 (1943).

(28) F. H. Johnson, E. A. Flagler, R. Simpson and K. McGeer, *J. Cell. Comp. Physiol.*, **37**, 1 (1951).

(29) M. Jowett, *J. Physiol.*, **92**, 322 (1938).

phenol disinfection of *Staphylococcus pyogenes aureus*[30,31]; inhibition of horse serum cholinesterase by alkyl fluorophosphates[32]; malonate inhibition of the succinic dehydrogenase of *Escherichia coli*[33]; inhibition of embryonic heart beat by cyanide[34]; inhibition of yeast respiration by ethyl carbamate[35]; azide inhibition of *Cypridina* luciferase.[36] Many similar examples from the older literature are discussed by Clark.[37]

This practice, though empirical in origin, implies a relationship of the type

$$\ln E_t = m \ln i^0 + \ln C$$

Substitution of k_2/k_x or k_2/k_y for m in this expression gives a result identical with eq. (14) or eq. (22). This identity satisfies the need, expressed by Hinshelwood,[38] for a rational interpretation of the arbitrary coefficient, m. The validity of eq. (14) and (22) is thus supported by the widespread, successful use of $\ln E_t$ *vs.* $\ln i^0$ plots,[39] in which the coefficient m can now be identified as the ratio of two rate constants: one, a characteristic of the enzyme system alone; the other, a characteristic of the inhibitor *and* the enzyme system. This point supports the earlier contention that synergism is as much a property of the enzyme system as it is a property of the inhibitors.

Equation (27) is the defining equation for synergism. The locus of eq. (27), when converted to its exponential form, is identical in all essential respects to a boloform surface of the type found experimentally in cases of synergism. When $k_2 > k_x$ and $k_2 > k_y$ this surface has the general shape shown in Fig. 1, adapted from Loewe.[7] When $k_2 < k_x$ or $k_2 < k_y$, the individual dose-effect curves, AA′ or BB′, have curvatures opposite to those shown, but—as will appear later—the direction of curvature of isoboles AB and A′B′, which is the criterion for synergism, is the same for all combinations of the values of k_2, k_x and k_y.

Experimental application of eq. (27) is most convenient when one of the concentration variables is held constant; the resulting graph is then a vertical profile of the boloform surface. A profile of this type is also linear in $\ln E_t$ *vs.* $\ln i^0$ since the constancy of the *fixed* inhibitor concentration becomes a part of $\ln C$. The use of eq. (27) in this manner is particularly convenient in the *in vitro* study of inhibited bacterial growth because of the simple proportionalities relating E_t, n_t and A_t, as expressed by definitions (j) and (f).[40] For this purpose, eq. (27) becomes

$$-\ln A_t = \frac{k_2}{k_x} \ln i_1^0 + \frac{k_2}{k_y} \ln i_2^0 + \ln C$$

in which the proportionality constants in definitions (j) and (f) are part of new intercept, $\ln C$. Applicability of eq. (27) is not, of course, limited to bacterial growth.

Equation (27) has a number of properties of interest for their bearing on the concept of synergism. The multiplicative effect of joint inhibition is, in principle, most easily seen for the special case

$$k_2 = k_x = k_y$$

for eq. (27) then takes the clearly multiplicative form

$$E_t = C i_1^0 i_2^0$$

For the more general and more likely case, $k_2 \neq k_x \neq k_y$, the function is more complicated, but in no case can it be additive for any combination of values of the quantities in eq. (27).

The practical relevance of eq. (27) to synergism is best seen, however, if it is given a geometric interpretation in terms of the combinations of i_1^0 and i_2^0 that lead to a constant-effect end-point; the result is a horizontal profile of Fig. 1 with analytic properties identical with those of an experimental isobole.[41] Thus, rearranging eq. (25) and substituting dE_t/E_t for $-dR_t/R_t$ (definition f) gives

$$\frac{dE_t}{E_t} = \frac{k_2}{k_x} \times \frac{di_1^0}{i_1^0} + \frac{k_2}{k_y} \times \frac{di_2^0}{i_2^0} \tag{31}$$

Since every point on an isobole refers to constant effect, $dE_t = 0$ and eq. (31) becomes

$$\frac{di_1^0}{di_2^0} = -\frac{k_x}{k_y} \times \frac{i_2^0}{i_1^0} \tag{32}$$

This is another variant of the allometric equation referred to earlier.[25,26] Integration of eq. (32) gives a form linear in $\ln i_1^0$ *vs.* $\ln i_2^0$

$$\ln i_1^0 = -\frac{k_x}{k_y} \ln i_2^0 + \ln C \tag{33}$$

or its equivalent

$$i_1^0 = C/(i_2^0)^w \tag{34}$$

where $w = k_x/k_y$. The 2nd-order analog of eq. (32) can be obtained by setting $dE_t = 0$ in eq. (30), leaving

$$\frac{di_1^0}{di_2^0} = -\frac{k_x}{k_y} \times \frac{e_1}{e_2} \times \frac{i_1^0}{i_2^0} \tag{35}$$

As expected, e_1 and e_2 appear explicitly, and the 1st-order result, eq. (34), is obtained when the 1st-order condition, $e_1 \sim e_2 >>> i_1^0 \sim i_2^0$, is imposed on eq. (35).

Equation (34) describes a family of curves, some segments of which are indicated in Fig. 2. A given curve is completely determined for particular values of C and w and the result can be considered a theoretical isobole for synergism between a pair of drugs, I_1 and I_2, that react in a 1st-order manner (rate constants k_x and k_y) with E_1 and E_2 in competition with S_1 and S_2, respectively. Each such isobole is a constant-effect profile of the theoretical boloform surface for the drugs in question within the concentration range for which synergism is demonstrable. The relationship of a theoretical isobole to a generalized boloform surface and to the individual dose-effect curves is shown in Fig. 1.

Each theoretical isobole of the type expressed by eq. (34) approaches the axes asymptotically with a slope that is everywhere negative. At the point of intersection of the line, $i_1^0 = i_2^0$, with an isobole

$$di_1^0/di_2^0 = \text{slope} = -w \tag{36}$$

but, in general

$$-w = \text{slope} \times (i_2^0/i_1^0) \tag{37}$$

(30) H. Chick, *J. Hyg.*, **8**, 92 (1908).
(31) H. E. Watson, *ibid.*, **8**, 536 (1908).
(32) J. F. Mackworth and E. C. Webb, *Biochem. J.*, **42**, 91 (1948).
(33) J. H. Quastel and W. R. Wooldridge, *ibid.*, **22**, 689 (1928).
(34) K. C. Fisher and R. Ohnell, *J. Cell. Comp. Physiol.*, **16**, 1 (1940).
(35) K. C. Fisher and J. R. Stearn, *ibid.*, **19**, 109 (1942).
(36) A. M. Chase, *ibid.*, **19**, 173 (1942).
(37) A. J. Clark, "General Pharmacology," in "Heffter's Handbuch der experimentellen Pharmakologie," W. Heubner and J. Schüller, eds., Verlag von Julius Springer, Berlin, 1937, Bd. IV.
(38) C. N. Hinshelwood, ref. 16, pp. 101-102.
(39) See also the adsorption function of A. J. Clark, ref. 37, p. 38.
(40) See also M. Kurokawa, M. Hatano, N. Kashiwagi, T. Saito, S. Ishida, and R. Homma, *J. Bact.*, **83**, 14 (1962).
(41) L. G. Goodwin, see ref. 9.

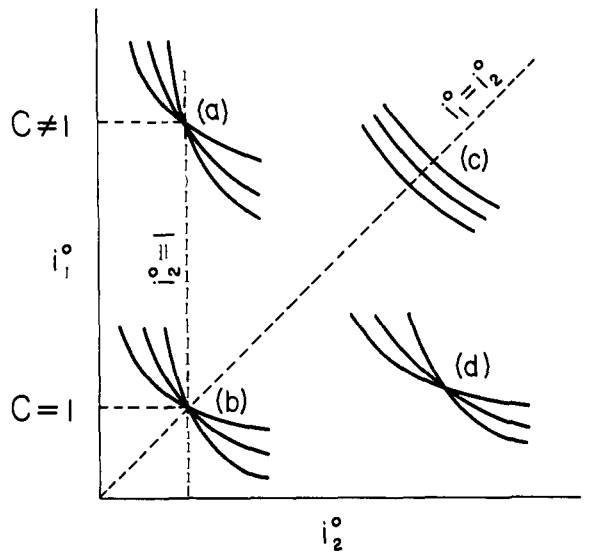

Fig. 2.—Segments of the theoretical isobole, $i_1^0 = C/(i_2^0)^w$, for various values of C and w: (a) same C ($\neq 1$), different w's; (b) same C (= 1), different w's; (c) different C's, same w; (d) different C's, different w's.

by rearrangement of eq. (32). The symmetry of a theoretical isobole relative to the line $i_1^0 = i_2^0$ is thus determined by the magnitude of w; for the special case, $w = 1$, the isobole is a rectangular hyperbola.[41a]

Differentiation[42] of eq. (32) gives

$$\frac{d^2 i_1^0}{(di_2^0)^2} = (w^2 + w)\frac{i_1^0}{i_2^0} \tag{38}$$

For all meaningful values of i_1^0, i_2^0, and w in eq. (38)

$$d^2 i_1^0/(di_2^0)^2 > 0 \tag{39}$$

so every isobole is concave as shown, as required for synergism and as found experimentally.

A similar test for the direction-of-curvature of the 2nd-order isobole, obtained by partial differentiation of eq. (35), gives a result identical with eq. (39); this identity is another indication of the qualitative equivalence of the 1st- and 2nd-order treatments.

The ratio, w, can be found from eq. (37) by visually fitting a tangent, di_1^0/di_2^0, to any point, (i_1^0, i_2^0), on an isobole. This is more easily done, however, from a linear plot of ln i_1^0 *vs.* ln i_2^0, from which $(-w)$ = slope directly, as required by eq. (33). C, in a logarithmic plot of eq. (33), is the extrapolated value of i_1^0 for $i_2^0 = 1$.

The asymptotic approach of each end of a theoretical isobole, eq. (34), to the axes implies that, contrary to experience, an infinite concentration of either drug alone would be required to produce the standard response; this feature, though inconvenient, is in itself only a formal detriment to the theory,[43] for the same limitation is inherent in the kinetics analysis of any 1st-order reaction. Thus, for

$$A \xrightarrow{k} B$$

the rate equation, $-dA/dt = kA$, is indeterminate in t for $A = 0$, but first-order reactions do, in practice, go to completion in finite time. The indeterminate forms of eq. (33) and (34) for $i_1^0 = 0$ or $i_2^0 = 0$ thus result from the assumption of a 1st-order mechanism.

The 2nd-order result, eq. (35), would presumably also lead to an indeterminate form, but this cannot be established formally since eq. (35) cannot be integrated under 2nd-order conditions. The 1st-order theoretical isobole, eq. (34), can be given meaningful intercepts, however, by translation to a new coördinate system with a new origin at (b,a). This is a valid, indeed, a realistic procedure since drugs must often exceed certain threshold concentrations (b or a) before any effect is observed.[38,43] Letting a and b represent the threshold concentrations for I_1 and I_2, respectively, eq. (33) can be translated to the new coördinate system

$$i_1^0 = x + a$$

$$i_2^0 = y + b$$

where x and y are the concentrations of I_1 and I_2 in excess of threshold concentrations, a and b. b and a are both constants, so the new origin is (b,a) and eq. (33) becomes

$$\ln (x + a) = -w \ln (y + b) + \ln C \tag{40}$$

Intercept, ln C, is now the value of ln $(x + a)$ for $(y + b) = 1$.

The result of this translation of coördinates is, in isobole form

$$(x + a) = C/(y + b)^w \tag{41}$$

with a general shape shown in Fig. 3. The new isobole,

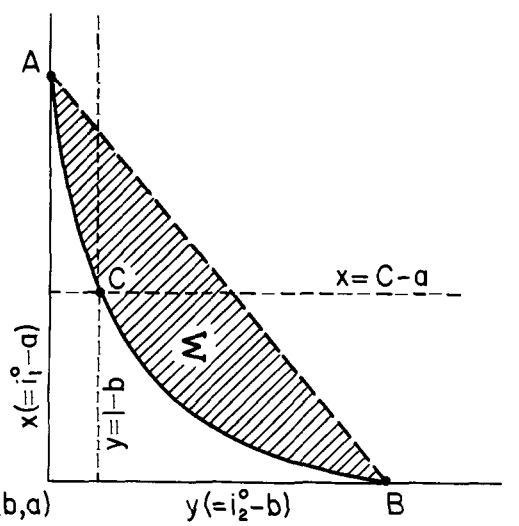

Fig. 3.—Graph of the function $(x + a) = C/(y + b)^w$ relative to an origin at (b,a); a theoretical isobole in translated coördinates.

now plotted on an x *vs.* y scale, is of course identical with the previous one, but relative to the new origin (b,a), and has finite intercepts on the x and y axes. C and $(-w)$ are best determined from eq. (40) by a linear plot of ln $(a + x)$ *vs.* ln $(b + y)$.

The intercepts, A and B, in Fig. 3 are now finite, for if, in eq. (41), $y = 0$, then $x = A = (C/b^w) - a$, and if $x = 0$, then

$$y = B = (C/a)^{k_y/k_x} - b$$

Intercept A is the concentration of drug I_1 in excess of its minimum effective concentration (a) that, when act-

(41a) R. A. Edgren, *Ann. N.Y. Acad. Sci.*, **83**, 170 (1959), has referred to the hyperbolic nature of experimentally established isoboles.

(42) By logarithmic differentiation as described by G. J. Kynch, ref. 24, p. 48.

(43) A. J. Clark, ref. 37, p. 7.

ing in the presence of a threshold concentration (b) of I_2, causes the standard response. The meaning of intercept B is clear by analogy.

Note that Fig. 1 is plotted relative to the same translated coördinate system, and that the E_t-coördinate of the origin, $(E_t)_{b,a}$, is therefore not zero; it must be found by translation of eq. (27) and casting the result into exponential form, giving

$$E_t = C(a + x)^{k_2/k_x}(b + y)^{k_2/k_y} \quad (42)$$

whence, by setting $x = 0$ and $y = 0$

$$(E_t)_{b,a} = Ca^{k_2/k_x} b^{k_2/k_y} \quad (43)$$

The individual dose-effect curves (BB′ and AA′) in Fig. 1 are defined by eq. (42) when $x = 0$ or $y = 0$.

In theory, a threshold-effect isobole should connect points b and a in the $E_t = 0$ plane of a boloform surface. This predicts the existence of synergism when $i_1^0 = ga$ and $i_2^0 = hb$, g and h being fractions such that $g + h < 1$. This isobole is not seen, however, in Fig. 1 since the $E_t = 0$ plane is not shown there.

The cross-hatched area between the additivity and synergism isoboles in Fig. 3 has been defined as the "amount" of synergism.[44] This quantity, Σ, can be determined directly from the graph by mechanical integration or by integration of eq. (41). Letting 0 represent the origin, then

$$\text{Area AOB} = (\text{AB})/2$$

and

$$\text{Area AOCB} = C\int_0^B (y + b)^{-w}dy - a\int_0^B dy$$

$$\Sigma \equiv \text{AOB} - \text{AOCB}$$

so

$$\Sigma = (\text{AB})/2 - \left(C\int_0^B (y + b)^{-w}dy - a\int_0^B dy\right)$$

When $w \neq 1$, integration gives

$$\Sigma = (\text{AB})/2 - \frac{C}{(1 - w)(B + b)^{(w-1)}} + \frac{C}{(1 - w)b^{(w-1)}} + aB$$

For the special case, $w = 1$, the integration gives

$$\Sigma = (\text{AB})/2 - C\ln(B + b) + C\ln b + aB$$

Σ is thus determined entirely by the graphical constants for a given system.

Acknowledgments.—I have benefited from many discussions of this problem with many of my associates; among them, I owe particular thanks to Mr. Leonard Doub who first aroused my interest in the subject and offered many valuable criticisms of the manuscript during its preparation. I am also grateful to the referees of this paper for citing three references[46,47a,47b] which contribute to the record and have, therefore, been added. These references, though relevant to the present work, do not in any way anticipate its methods or conclusions.

(44) C. B. Elion, S. Singer and G. H. Hitchings, *J. Biol. Chem.*, **208**, 477 (1954).

(45) J. R. Fouts and B. B. Brodie, *J. Pharmacol. Exper. Therap.*, **116**, 480 (1956).

(46) J. H. Gaddum, *Pharmacol. Revs.*, **9**, 211 (1957).

(47) (a) S. E. de Jongh in, "Quantitative Methods in Pharmacology," H. de Jonge, ed., North-Holland Publ. Co., Amsterdam, 1961, pp. 318–327; (b) P. S. Hewlett and R. L. Plackett, *ibid.*, pp. 328–339.

Appendix

Derivation of Equation (28).—The procedure is analogous to that followed for the 1st-order model except that e_1 and e_2 must be treated as variables and freer use of partial differentiation must be made in handling the relevant definitions, (f), (i), (k)–(n).

From these definitions

$$R_t = f(e_2, s_2) \quad (A1)$$

$$s_2 = f(s_1) \quad (A2)$$

$$s_1 = f(i_1) \quad (A3)$$

and

$$i_1 = f(i_1^0) \quad (A4)$$

So, by theorem B

$$\frac{dR_t}{di_1^0} = \frac{dR_t}{ds_2} \times \frac{ds_2}{ds_1}\left(-\frac{ds_1}{di_1}\right)\frac{di_1}{di_1^0} \quad (A5)$$

In the strictest sense, eq. (A2)–(A4) and the operators in eq. (A5) should be written as partial dependencies; it will be seen later, however, that certain convenient cancellation properties require that only R_t be treated explicitly as a function of two variables, as specified in eq. (A1.)

Applying theorem A to eq. (A1) gives

$$dR_t = \left(\frac{\partial R_t}{\partial s_2}\right)_{e_2} ds_2 + \left(\frac{\partial R_t}{\partial e_2}\right)_{s_2} de_2 \quad (A6)$$

and these operations, applied to definition (k), give

$$dR_t = (k_2e_2)ds_2 + (k_2s_2)de_2 \quad (A7)$$

or

$$\frac{dR_t}{ds_2} = k_2e_2 + (k_2s_2)\frac{de_2}{ds_2} \quad (A8)$$

But $de_2/ds_2 = 1$, so eq. (A8) becomes

$$\frac{dR_t}{ds_2} = k_2e_2 + k_2s_2 \quad (A9)$$

Definition (k) divided by definition (l) is

$$\frac{ds_2}{ds_1} = \frac{k_2e_2s_2}{k_1e_1s_1} \quad (A10)$$

and definition (l) divided by definition (m) gives

$$-\frac{ds_1}{di_1} = -\frac{k_1e_1s_1}{k_xe_1i_1} = -\frac{k_1s_1}{k_xi_1} \quad (A11)$$

Integration of definition (m) gives

$$\ln i_1 = -k_x\int e_1dt + \ln i_1^0 \quad (A12)$$

or

$$i_1 = i_1^0e^{-k_x\int e_1dt} \quad (A13)$$

and partial differentiation of eq. (A13) gives

$$\frac{di_1}{di_1^0} = e^{-k_x\int e_1dt} \quad (A14)$$

Equations (A9), (A10), (A11) and (A14) are the terms in eq. (A5), so eq. (A5) becomes, after these substitutions

$$\frac{dR_t}{di_1^0} = (k_2e_2 + k_2s_2)\left(\frac{k_2e_2s_2}{k_1e_1s_1}\right)\left(-\frac{k_1s_1}{k_xi_1}\right)e^{-k_x\int e_1dt} \quad (A15)$$

Substituting definition (k) for $(k_2e_2s_2)$, eq. (A13) for i_1, cancelling identical terms, factoring, and rearranging gives

$$-\frac{dR_t}{R_t} = \frac{k_2}{k_x}\left(\frac{e_2 + s_2}{e_1}\right)\frac{di_1^0}{i_1^0} \quad (A16)$$

Substitution of definition (f) in eq. (A16) gives

$$\frac{dE_t}{E_t} = \frac{k_2}{k_x}\left(\frac{e_2 + s_2}{e_1}\right)\frac{di_1^0}{i_1^0} \quad (28)$$

Derivation of Equation (29).—As in the 1st-order case for inhibition by I_2 alone

$$\frac{dR_t}{di_2^0} = \frac{dR_t}{ds_2}\left(-\frac{ds_2}{di_2}\right)\frac{di_2}{di_2^0} \quad \text{(A17)}$$

But

$$R_t = f(s_2,e_2) \quad \text{(A18)}$$

so, by theorem A

$$dR_t = \left(\frac{\partial R_t}{\partial s_2}\right)_{e_2} ds_2 + \left(\frac{\partial R_t}{\partial e_2}\right)_{s_2} de_2 \quad \text{(A19)}$$

or

$$\frac{dR_t}{ds_2} = \left(\frac{\partial R_t}{\partial s_2}\right)_{e_2} + \left(\frac{\partial R_t}{\partial e_2}\right)_{s_2}\frac{de_2}{ds_2} \quad \text{(A20)}$$

The dependence of R_t on i_2 is omitted in eq. (A18)–(A20) since it has already been acknowledged in the first two terms of the right-hand side of eq. (A17).

Since $de_2/ds_2 = 1$, eq. (A20) may be simplified, giving

$$\frac{dR_t}{ds_2} = \left(\frac{\partial R_t}{\partial s_2}\right)_{e_2} + \left(\frac{\partial R_t}{\partial e_2}\right)_{s_2} \quad \text{(A21)}$$

Applied to definition (k), eq. (A21) becomes

$$\frac{dR_t}{ds_2} = k_1e_2 + k_2s_2 \quad \text{(A22)}$$

Definition (k) divided by definition (n) gives

$$-\frac{ds_2}{di_2} = -\frac{k_2e_2s_2}{k_ye_2i_2} \quad \text{(A23)}$$

Integration of definition (n) gives

$$\ln i_2 = -k_y\int e_2dt + \ln i_2^0 \quad \text{(A24)}$$

or

$$i_2 = i_2^0e^{-k_y\int e_2dt} \quad \text{(A25)}$$

and partial differentiation of eq. (A25) gives

$$\frac{di_2}{di_2^0} = e^{-k_y\int e_2dt} \quad \text{(A26)}$$

Substitution of eq. (A22), (A23) and (A26) in eq. (A17) gives

$$\frac{dR_t}{di_2^0} = (k_2e_2 + k_2s_2)\left(-\frac{k_2e_2s_2}{k_ye_2i_2}\right)e^{-k_y\int e_2dt} \quad \text{(A27)}$$

Substituting definition (k) for $k_2e_2s_2$ and eq. (A25) for i_2, cancelling identical terms, factoring and rearranging gives

$$-\frac{dR_t}{R_t} = \frac{k_2}{k_y}\left(\frac{e_2 + s_2}{e_2}\right)\frac{di_2^0}{i_2^0} \quad \text{(A28)}$$

Substitution of definition (f) in eq. (A28) gives

$$\frac{dE_t}{E_t} = \frac{k_2}{k_y}\left(\frac{e_2 + s_2}{e_2}\right)\frac{di_2^0}{i_2^0} \quad \text{(29)}$$

Derivation of Equation (30).—By the reasoning given in the 1st-order case, eq. (28) and (29) may be combined directly to give eq. (30).

Monoamine Oxidase Inhibitors. IV. Some Dialkylaminophenylalkylhydrazines and Related Compounds

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Two sequences of chemical reactions leading to several new basically substituted phenylalkylhydrazines are described. The compounds were less active and/or more toxic than the unsubstituted parent compound in limited animal tests.

Certain aralkylhydrazines and selected acyl derivatives of them are potent, long-acting monoamine oxidase inhibitors.[1] Substitution of the phenyl ring with amino or dialkylamino residues converted the length of activity from periods of the order of 25 days to less than 1 day. It was noted[2] that with increasing length of the alkylene bridge, compounds of intermediate length of activity (4–5 days) were obtained. This paper describes the preparation of these latter materials.

(4-Dimethylamino-α-methylphenethyl)hydrazine (V) was synthesized as follows

$$p\text{-}O_2NC_6H_4CH_2CCCH_3 \rightarrow p\text{-}(CH_3)_2NC_6H_4CH_2COCH_3 \rightarrow p\text{-}(CH_3)_2NC_6H_4CH_2C(CH_3)=NNHCOCH_3 \rightarrow$$
$$\text{I} \qquad\qquad \text{II} \qquad\qquad \text{III}$$

$$p\text{-}(CH_3)_2NC_6H_4CH_2CH(CH_3)NHNHCOCH_3 \rightarrow p\text{-}(CH_3)_2NC_6H_{10}CH_2CH(CH_3)NHNHCOCH_3$$
$$\text{IV} \qquad\qquad \text{VI}$$

$$\downarrow \qquad\qquad \downarrow$$

$$p\text{-}(CH_3)_2NC_6H_4CH_2CH(CH_3)NHNH_2 \qquad p\text{-}(CH_3)_2NC_6H_{10}CH_2CH(CH_3)NHNH_2$$
$$\text{V} \qquad\qquad \text{VII}$$

1-(4-Nitrophenyl)-2-propanone (I) was prepared by treating 4-nitrophenylacetyl chloride with diethyl ethoxymagnesium malonate.[3] It was reduced readily in the presence of formalin to produce 1-(4-dimethylaminophenyl)-2-propanone (II) in 91% yield as a yellow distillable oil, which was treated with acetyl hydrazine to form the corresponding acetylhydrazone (III) which was reduced to IV in acetic acid by H_2/PtO_2, stopping the absorption of one equivalent of hydrogen. If the reduction was permitted to continue, the cyclohexyl compound VI was formed which, on deacetylation, gave VII. Deacetylation of IV gave V.

The synthesis of the higher homolog, 1-(4-dimethylaminophenyl)-3-hydrazinobutane and related products, is shown in the scheme at the top of the next page.

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