THE MECHANISM OF ENZYME-INHIBITOR-SUBSTRATE REACTIONS*

ILLUSTRATED BY THE CHOLINESTERASE-PHYSOSTIGMINE-ACETYLCHOLINE SYSTEM

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INTRODUCTION

The phenomenon of enzyme inhibition has become a subject for pharmacological study with the growing awareness that many, if not all drugs owe their action to an ultimate combination with intracellular, extracellular or cell-surface proteins. Clark's (1) classic exposition of the manner in which drugs act upon living cells to alter their structure and function outlines the guiding principles for such investigation. More recently, the increasing interest in the mode of action of numerous bacteriostatic compounds has furthered an appreciation of the importance of enzyme inhibition as the underlying common denominator in the action of a variety of drugs. Fundamentally, this common denominator is probably not the inhibition of enzymes, as such, but rather the more general combination of a small molecule with a protein (not necessarily an enzyme), the resulting alteration of whose properties causes a change in the physiology of the cell or organism as a whole. Elucidation of the laws governing these general reactions may presumably be furthered through study of the mechanism of one type of such combination—that between enzyme and inhibitor. The pharmacologist must study such a reaction not alone from the standpoint of its inherent mechanism, but also with the realization that if he has abstracted the system from the animal to the test-tube, he must replace it again in its normal physiological environment. In other words, he must seek solutions not alone for the problems which arise under the artificial conditions of experimentation he has created, but also for the corresponding and more significant problems arising from action of the system *in vivo*. It is also true that results *in vitro* rarely will apply *in vivo* unless the artificial conditions set up in the experiment are unravelled, and appropriate changes made to fit the data obtained to the physiology of the living organism.

Straus and Goldstein (2) attempted to apply such a treatment to enzyme-

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inhibitor systems. On the basis of the mass law principles governing the reversible combination of enzymes and inhibitors, they were able to show some of the errors entailed in applying *in vitro* data to *in vivo* situations. They demonstrated that these systems could operate in three "zones of behavior", and that two of these are commonly neglected in treatments of enzyme kinetics even though they may be significant within the living cell or organism. They also showed that the common practice of diluting an enzyme-inhibitor system for determination is a crucial operation affecting the observed inhibition of the enzyme. Their theory yielded several other interesting conclusions which cannot be restated here. As the present studies are to some degree an extension and elaboration of the above mentioned work, the reader is referred to the original article for a comprehensive background of the material which is to follow.

The work of these authors, as they pointed out, was incomplete in several respects, two of which stand out prominently. *First*, it dealt only with *non-competitive* inhibition—that is, inhibition which is unaffected by the presence of substrate. Such a treatment could be applied *fully* to truly non-competitive inhibitors (although the system they chose for illustration was actually competitive), and also to enzyme-substrate combinations in which no third reactant is present; and *practically* to the case where competition, although present, is not significant. However, their treatment is not applicable to systems where substrate materially alters the enzyme-inhibitor equilibrium. *Second*, it confined itself to the study of equilibrium conditions and did not consider the attainment of equilibrium, which is a kinetic process. Time can be a very important factor and the formal application of their results based on equilibrium to a dynamic, transitional situation might be fraught with error.

It will be the primary purpose of this paper to consider the two major problems omitted from the previous work, and further to develop the usefulness of the zone analysis of enzyme behavior. Emphasis will be placed upon the theoretical and general rather than upon the experimental and specific, but experimental data will be introduced frequently for purposes of illustration. Such data will refer to the cholinesterase-physostigmine-acetylcholine system which was also used by Straus and Goldstein; we shall therefore be able to demonstrate for a particular system the practical effects of incompleteness in their theoretical postulates. We wish to emphasize that nothing in our analysis limits its validity to any single system and it is to be hoped that the analytical methods elaborated here will be applied to others of similar type.

Zone Behavior in Competitive and Non-Competitive Systems

Non-Competitive Equilibrium.—

Let us consider a reversible enzyme-inhibitor combination in which sub-

strate plays no part—the non-competitive case represented by the reaction:

$$E + I \rightleftharpoons EI.^*$$

The activity of the enzyme at any time depends upon its ability to combine with a substrate. The degree of inhibition will be equal to the fraction of total enzyme centers prevented from combining with substrate, and conversely the degree of activity will be the fraction remaining free. We may designate this *fractional activity* by a, and define $a \equiv E_f/E$, where E_f and E are free and total enzyme respectively. At equilibrium we may write the mass law expression:

$$\frac{(E_f)(I_f)}{(EI)} = K_I$$

or

$$\frac{(E_f)(I - EI)}{(EI)} = K_I \qquad (1)$$

* *Table of Symbols Used*

Symbol	Definition	Refer to page:
E, E_f	Molar concentration of *total* and *free* enzyme centers	531
I, I_f	Molar concentration of *total* and *free* inhibitor	532
S, S_f	Molar concentration of *total* and *free* substrate	532–33
EI	Molar concentration of enzyme-inhibitor complex	532
ES	Molar concentration of enzyme-substrate complex	532
K_I, K_S	Dissociation constants of enzyme-inhibitor and enzyme-substrate complex	531–32
I', S'	Specific concentrations of inhibitor and substrate, defined as I/K_I and S/K_S respectively	532–33
E'_I, E'_S	Specific concentration of enzyme, in terms of inhibitor and of substrate, defined as E/K_I and E/K_S respectively	532–33
a	Fractional activity of enzyme, defined as E_f/E in non-competitive and ES/E in competitive system. See also discussion on p. 539	531
i	Fractional inhibition of enzyme, defined as EI/E	539
v	Observed velocity of destruction of a substrate	533
$V_{max.}$	Maximal velocity of substrate destruction for a given enzyme concentration	533
n	Molecules of substrate or inhibitor combining with each enzyme center	549
k_D	Velocity constant for destruction of a substrate	533
k_1, k_2	Forward and reverse velocity constants for the enzyme-inhibitor reaction	562
k_3, k_4	Forward and reverse velocity constants for the enzyme-substrate reaction	562
N	Factor of dilution; <1 for diluting a system, >1 for concentrating	554
t	Time in minutes	

Zones are defined as A, B, C, with respect to inhibitor or substrate in non-competitive systems (A_I, B_I, C_I, A_S, B_S, C_S) and to both in competitive systems (A_IA_S, A_IB_S, etc.).

Special Symbols Used in the Section on Destruction:

b	Initial molar concentration of physostigmine (equivalent to I)	573
x	Amount of physostigmine destroyed in time t	573
a	Molar concentration of second reactant (either E or hydroxyl)	573
c	An arbitrary proportionality constant	574

where I_f and I represent concentrations of free and total inhibitor, and EI the concentration of enzyme-inhibitor complex. Since $E = E_f + EI$ and $E_f = aE$, it follows that $EI = E(1 - a)$. Substituting this value we find that

$$I = K_I \cdot \frac{(1 - a)}{a} + (1 - a)E \tag{2}$$

Generalizing the equation to apply to any system, we eliminate K_I and introduce the terms $I' \equiv I/K_I$ and $E_I' \equiv E/K_I$, referred to as *specific* concentration of inhibitor and enzyme respectively ((2), page 563). Equation 2 then becomes

$$I' = \frac{(1 - a)}{a} + (1 - a)E_I' \tag{3B}$$

These equations are entirely identical with equations 2 and 3 of Straus and Goldstein except that for certain reasons which will be discussed below, we have used a term a, to represent fractional activity, instead of their fractional inhibition i; here, $a = (1 - i)$.

It will be recalled that zones of behavior are established on the basis of equation $3B$. It states that the total inhibitor I' is composed of two separate parts: $(1 - a)E_I'$ which is equal to $(EI)_I'$, the *combined* inhibitor; and $(1 - a)/a$, which must consequently represent the *free* inhibitor. Zone A is that zone in which essentially all the inhibitor is free and

$$I' = (1 - a)/a \tag{3A}$$

In zone B the full equation must be used, while zone C is that region in which practically all the inhibitor is combined and

$$I' = (1 - a)E_I' \tag{3C}$$

The legitimacy of using $3A$ or $3C$ instead of the full equation obviously depends upon the magnitude of E_I' and boundary values of E_I' can be established, within which either simplified form may be used without exceeding any agreed upon error Δa in the dependent variable. For $\Delta a = 0.01$ the approximate boundary values of E_I' are as follows: For the boundary AB, $E_I' = 0.1$; for the boundary BC, $E_I' = 100$. (See Fig. 1 of Straus and Goldstein.)

In treating the reaction of substrate with enzyme

$$E + S \rightleftarrows ES \rightarrow E + \text{split products}$$

we must define a as equal to ES/E, since enzyme activity is directly observed by measuring the rate of destruction of substrate, which rate is proportional to the concentration of complex (ES). The term a is therefore also equal

to the ratio of observed velocity to the maximum velocity which would be attained if the enzyme were saturated with excess substrate, since,

$$v = k_D(ES)$$

$$V_{max.} = k_D E$$

$$\frac{v}{V_{max.}} = \frac{ES}{E} \equiv a$$

where k_D is the velocity constant for destruction of substrate.

Proceeding exactly as in the derivation of equation 3, we arrive at an equation which is almost identical with $3B$:

$$S' = \frac{a}{1 - a} + aE_S' \tag{4B}$$

where, of course, $S/K_S \equiv S'$ and $E/K_S \equiv E_S'$. This equation follows exactly the same zone principles already outlined and is in fact the simplest type of reaction to analyze in terms of zone behavior; for it is entirely non-competitive and also subject to none of the errors to be discussed in connection with more complex systems. It is to be noted here that in the above derivations and throughout this paper we have considered *concentrations* of enzyme, inhibitor, and substrate as equivalent to their *activities*. Actually the mass law equations call for the use of the latter; and furthermore it is quite likely that for charged molecules (enzymes and many inhibitors and substrates) activity coefficients will vary with concentration. However information on this subject is still too incomplete to allow its inclusion in the present mathematical treatment.

What can we say regarding the real likelihood that enzyme-substrate systems will operate in one zone or another; *i.e.*, that they will be described well by either simplification or by the full equation? Since $E_S' \equiv E/K_S$, it follows that if K_S is not exceptionally small, and since the molar concentration of the protein enzyme cannot be very great (at least *in vitro*), E_S' is very likely to be less than 0.1. In that case the system will operate in zone A. That this is generally true is indicated by the fact that the classical treatment of enzyme-substrate systems (Michaelis and Menten (3)) has satisfactorily employed the zone A equation,

$$S' = a/(1 - a) \tag{4A}$$

It must be borne in mind, however, that the above conditions for zone A behavior need not apply under all conditions nor for all substrates and enzymes.

In Fig. 1 we present a typical "dose-effect curve" in which the activity of an enzyme is plotted against the $\log_{10}$ of the substrate concentration. We do not believe that the manner of portraying such experimental data is a question

to be decided by individual preference, since if clarity and ease of analysis are furthered by a particular type of plot, it should obviously be generally adopted. Yet it is interesting to find that the plot of Fig. 1, although familiar and advantageous in many respects, has not been universally accepted for describing a "dose-effect" relationship. Its advantages are: (1) it corresponds with ex-

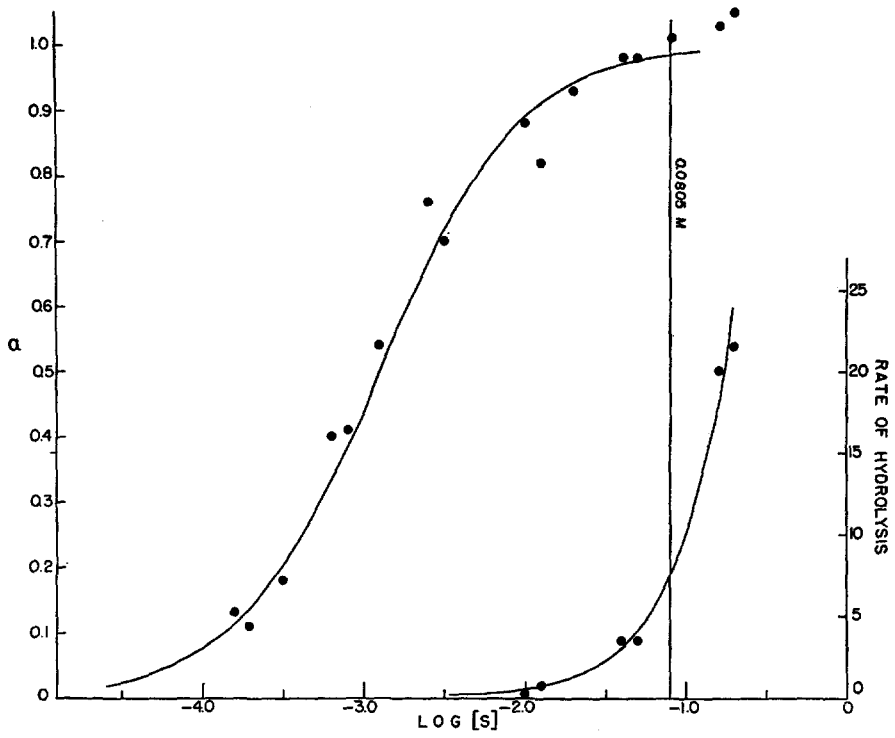

FIG. 1. Acetylcholine activity curve. Ordinate (left): fractional activity, *a*. Ordinate (right): non-enzymatic hydrolysis rate (cubic millimeters CO_2 per 20 minutes). Abscissa: $\log_{10}$ of the molar acetylcholine concentration. The solid vertical line shows the standard substrate concentration used in the other experiments described.

● = averages of two or more experimental determinations.

perimental conditions in that the abscissa and ordinate respectively represent the actual independent and observed dependent variable; (2) it shows clearly the *asymptotic* nature of the typical "dose-effect" mechanism; (3) it depicts the entire range from 0 to 1 in the fractional activity of an enzyme without distortion; (4) it lends itself to a mathematical analysis in which its shape, slope, and position assume real significance—this point will be amplified in a later section.

The data for Fig. 1 are obtained from experiments in which the concentration of acetylcholine was varied and the resulting changes in velocity of acetylcholine destruction observed. (See page 560 for experimental method.) If the usually employed zone A equations are valid in this case, it is a comparatively simple matter to solve for $V_{max.}$ and K_S from the relation

$$\frac{1}{v} = \frac{K_S}{V_{max.}} \cdot \frac{1}{S} + \frac{1}{V_{max.}} \tag{5}$$

modified from Michaelis and Menten, who considered only the zone A case (E does not enter the equation). Substitution into this equation gives $K_S = 1.25 \times 10^{-3}$, an acceptable value if the system really lies in zone A. That it does is indisputable in the present instance, for we know from other experiments (pages 543 and 544) that E is of the order of 10^{-8} molar or less. For a system to operate in zone B (E' greater than 0.1) when K_S is as large as 10^{-3} would require a molar enzyme concentration of at least 10^{-4}. Accepting the results obtained from equation 5 we may then express activities as $v/V_{max.} = a$ and plot the experimental points accordingly. We may also draw the theoretical zone A curve with $K_S = 1.25 \times 10^{-3}$ (the solid line of Fig. 1), to which the experimental points are seen to fit satisfactorily. This value of K_S agrees well with Glick's (4) 1.1×10^{-3} and Eadie's (5) 1.7×10^{-3}, and will be used throughout this paper.

The method just described for fitting a curve to the experimental points may seem excessively involved and argumentative when strictly mathematical means might better be employed. However, we wish to emphasize the contrary view that here careful reasoning is superior to the formal use of statistical methods. The generally accepted method for curve-fitting to the zone B equation is that proposed by Easson and Stedman (6) and used since by a number of authors including Straus and Goldstein. Their procedure is to divide the full equation by a or $(1 - a)$ so that

$$\frac{S}{a} = K_S \cdot \frac{1}{1 - a} + E$$

and

$$\frac{I}{1 - a} = K_I \cdot \frac{1}{a} + E$$

One can then plot S/a against $1/(1 - a)$ or $I/(1 - a)$ against $1 - a$ and obtain a linear curve with slope equal to K and intercept equal to E. From these constants one can construct the theoretical curve with the desired abscissa ($\log I$) and ordinate (a).

But unfortunately the transformation of variables alters the equation in such a way that the curve plotted from the resulting constants may not be the

best fit to the experimental data. This is because the best fit of a linear curve to the transformed equation is *not* the same as the best fit of the original hyperbolic function to the experimental points. This is emphasized by the vastly disproportionate weights of the points at one end of the curve (when a is small in the equation for I; when a is large in the equation for S). When one fits such a linear curve to the transformed data by means of a method of least squares (7), one is impressed by the fact that all the experimental points but a few at the crucial end of the curve could be omitted entirely with little effect on the end-result. For example, in the data for Fig. 1, a very small error in the points near $a = 1$ could so affect the constants obtained by this method that E would appear large enough to place the system in zone B. Although such a conclusion would be derived from an apparently legitimate mathematical method, it would be, as we have shown, nonetheless untenable; and the directly plotted zone A curve of Fig. 1 would be a far more truthful portrayal of the experimental data.

On account of the inherent faults in this statistical method, it is preferably not used at all, or employed with caution so as to avoid if possible the distortions which can so easily occur. Since the absolute experimental error is the same for all values of a, a curve-fitting method is required which would weight all points equally so that the standard deviation of the experimental points (*not of the transformed points*) would be minimal from the desired curve.

Although we have introduced a term $V_{max.}$, it is important to realize that "maximal velocity" is unobtainable in this type of reaction, just as "complete inhibition" is a misnomer when an inhibitor is the independent variable. These reactions, as we have pointed out, are all asymptotic to some hypothetical value which is never actually (although it may be practically) attained. Consequently one cannot conceivably use an "excess" of substrate to "saturate" an enzyme, except in a very figurative sense. This seems to be repeating the obvious, but it may not be generally realized that significant errors can arise from the notion that a particular substrate concentration is adequate to produce a "maximal" rate of enzyme activity.

Fortunately one can predict from the zone A equation what the value of a must be for a given concentration of S, once K_S is known for any substrate. We have, for example, used 0.0805 M acetylcholine in all our studies requiring constant S and variable I; this is equivalent to $S' \equiv S/K_S = 64.4$. Reference to equation 4A or Fig. 1 will show that this concentration ($\log S' = 1.81$) gives $a = 0.985$; that is to say, 98.5 per cent of the enzyme is saturated. Although this is quite satisfactory from a practical standpoint, casual perusal of the literature reveals substrate concentrations in general use which must result in considerably less complete enzyme saturation, no longer even approaching "maximal." The errors entailed in such experiments will be taken up in the

appropriate section dealing with suboptimal substrate concentration (page 545). We suggest that in the case of a new substrate, several quick determinations with varying concentrations of S can yield a rough figure for K_S (that concentration of S giving half the apparent maximal velocity). Then equation $4A$ will give the necessary S to achieve any desired saturation of the enzyme.

The curve at the lower right of Fig. 1 shows the non-enzymatic hydrolysis of acetylcholine as a function of acetylcholine concentration. It is a convenient coincidence that the substrate concentration we have chosen to use is just below that producing a sharp rise in the hydrolysis curve, so that although we always correct for hydrolysis, this correction remains a small one.

Competitive Equilibrium.—

By *competitive* equilibrium we mean one into which enter not only enzyme and inhibitor or enzyme and substrate, but all three elements simultaneously. Thus two separate equilibrium equations must be satisfied.

$$\frac{(E_f)(I_f)}{(EI)} = K_I \quad \text{and} \quad \frac{(E_f)(S_f)}{(ES)} = K_S$$

As before, let $(ES)/E \equiv a$ so that $(ES) = aE$. Then since $E = ES + EI + E_f$,

$$\frac{EI + E_f}{E} = (1 - a) \quad \text{and} \quad EI = (1 - a)E - E_f.$$

For (E_f) we may substitute its value $\frac{K_S(ES)}{S_f} = \frac{K_S aE}{S - aE}$.

For (I_f) substitute $(I - EI)$, and for (S_f), $(S - ES)$.

And introducing $I' \equiv I/K_I$, $S' \equiv S/K_S$, $E_I' \equiv E/K_I$ and $E_S' \equiv E/K_S$, we have:

$$I' = \underbrace{\left[(S' - aE_S')\left(\frac{1 - a}{a}\right) - 1\right]}_{\text{Free}} + \underbrace{\left[1 - a\left(1 + \frac{1}{S' - aE_S'}\right)\right]E_I'}_{\text{Combined}} \qquad (6B_I B_S)$$

Total

It can be verified that the last expression is equal to $(EI)'$, the specific concentration of *combined* inhibitor. Reasoning as previously, we conclude that the first term on the right must be equal to *free* inhibitor. This is the equation describing competitive equilibrium when the system is in zone B with respect to both inhibitor and substrate. It is the most rigid form possible, since no approximations or simplifications were employed in its derivation.

The most direct simplifications to consider are those based on the equation already presented. If practically all the inhibitor is free, we may write:

$$I' = (S' - aE_S')\left(\frac{1 - a}{a}\right) - 1 \qquad (6A_I B_S)$$

And likewise, if practically all is combined:

$$I' = \left[1 - a\left(1 + \frac{1}{S' - aE_S'}\right)\right]E_I' \qquad (6C_I B_S)$$

The more commonly used simplifications will concern S rather than I for in general it will be common to find the enzyme operating in zone A with respect to substrate ($E/K_S < 0.1$) but in another zone with respect to inhibitor (since K_S will often be much greater than K_I). In zone A_S, practically all the substrate is free, so that $S' - aE_S' \doteq S'$. This allows us to write:

$$I' = \left[S' \cdot \frac{1 - a}{a} - 1\right] + \left[1 - a\left(1 + \frac{1}{S'}\right)\right]E_I' \qquad (6B_I A_S)$$

and

$$I' = S' \cdot \frac{1 - a}{a} - 1 \qquad (6A_I A_S)$$

$$I' = \left[1 - a\left(1 + \frac{1}{S'}\right)\right]E_I' \qquad (6C_I A_S)$$

We must emphasize that the validity of these simplifications with respect to substrate depends *not* upon having an "excess" of S, in terms of concentration, but rather upon K_S and E being large enough or small enough so that $E_S' <$ 0.1, or $>$ 100, regardless of the actual concentration of S employed.

The case where practically all S is combined (zone C_S) would be represented by the equation

$$S' = aE_S' \qquad 4C$$

If this were true, a would be equal to the ratio S'/E_S' (or S/E, which is the same thing) regardless of the presence or absence of I. We would then have a valid non-competitive equation for zone C in an enzyme-substrate system, but no equation including inhibitor could be written.

Reinspection of equation 6 $C_I B_S$, which described zone C with respect to inhibitor, reveals a similar situation. If S' is very small, then a is limited to infinitesimal values and there is really no competitive inhibition, the equation itself reducing to the non-competitive form 3C. If, on the other hand, S' is large, competition is present but we are no longer in zone C, for a significant amount of I is displaced from combination to become *free* I, and the full equation (6 $B_I B_S$) must be used. It follows from this and the preceding paragraph that *competitive inhibition cannot* exist in zone C, *and, conversely, that if inhibition is competitive the zone must be* A *or* B.

A simplification of a different type is possible, if we agree to neglect (E_f) which must represent but a small fraction of the total enzyme when (EI) and

(ES) are both present and account for the major part of E. In the derivation we let $(ES)/E \equiv a$, as before, but now we let $EI/E \equiv (1 - a)$, so that (EI) is equal to $(1 - a)E$, instead of to $(1 - a)E - (E_f)$. Then by the same steps as before:

$$I' = (S' - aE_S')\left(\frac{1 - a}{a}\right) + (1 - a)E_I' \qquad (7B_I B_S)$$

Total Free Combined

The meaningful zone forms are then:

$$I' = (S' - aE_S')\left(\frac{1 - a}{a}\right) \qquad (7A_I B_S)$$

$$I' = S' \cdot \frac{1 - a}{a} + (1 - a)E_I' \qquad (7B_I A_S)$$

$$I' = S' \cdot \frac{1 - a}{a} \qquad (7A_I A_S)$$

The zone C form reduces, as we should expect, to the ordinary non-competitive equation $3C$.

This is a convenient time to return to a discussion of the term a which we have been using instead of the older i, introduced by Easson and Stedman, and used also by Straus and Goldstein. The reason for the change is quite basic and should be clear now that the various equations have been derived. In studying the activity of an enzyme, we can only observe its activity as reflected in its ability to act upon a substrate. The observed velocity is proportional to the concentration of enzyme-substrate complex $[v = k_D (ES)]$ so that a is both an experimental observation $(v/V_{max.})$ and a description of the ratio of enzyme-substrate complex to total enzyme (ES/E).[1] The term i, on the other hand, describes the fraction of enzyme-inhibitor complex $(i \equiv EI/E)$, of which we have no direct measure, so that we are forced to measure a experimentally and then substitute $i = (1 - a)$. We have just seen, moreover, that this is itself only an approximation which ignores free enzyme entirely. For the non-competitive case it was adequate, because the enzyme consisted of only two parts — EI, which was inactive; and E_f, which could be considered "active" by virtue of its ability to combine with a "saturating concentration" of substrate. We now see that in the competitive equilibrium a part of the enzyme may combine with neither inhibitor nor substrate. In fact, despite a negligible concentration of inhibitor the activity may nevertheless be very low

[1] For convenience in describing non-competitive enzyme-inhibitor systems we had to allow a to equal E_f/E (since no ES is present), but if this altered definition is remembered, no confusion should result.

because so little substrate is present. Therefore, since we are interested in enzyme *activity* and not in inhibitor-caused *inactivity*, we employ the term a, and suggest its general adoption.

Although a slight error is introduced (see below) we shall employ the simple forms of the equations in which (E_f) has been neglected. The equation which will find most general use is equation 7 $B_I A_S$ for reasons which have already been pointed out. This equation is identical in form with equation $3B$ except for the multiplier S'. The zone boundaries for equation $3B$ were derived by Straus and Goldstein and the steps need not be repeated here. In the non-competitive case the zone A form could be used when $E_I' < 0.1$. In the present case, then, the zone A form is valid when $E_I'/S' < 0.1$:

$$I' = S' \cdot \frac{1 - a}{a} \qquad \text{when } E_I'/S' < 0.1$$

$$I' = (1 - a)E_I' \qquad \text{when } E_I'/S' > 100.$$

Furthermore the "dose-effect" curves are identical for the competitive and non-competitive cases except for a shift on the log I' axis.

Fig. 2 is a plot of log I' as abscissa against a as ordinate for various values of E_I', showing the characteristic shapes and positions of the "dose-effect" curves in each zone. For the non-competitive case one ignores the term S' entirely so that the figure is then identical with Fig. 2 of Straus and Goldstein. It will be recalled that all values of $E_I' < 0.1$ are represented by a single symmetrical limiting zone A curve, inflecting at $a = 0.5$, and with slope 0.575 at that point. The curves for zone B become steeper and spaced out until in zone C, when $E_I' > 100$, they are parallel and essentially simple logarithmic curves (since $I' = (1 - a)E_I'$). To use these same curves for competitive inhibition in any system, one simply multiplies the value of E_I' by S' and I' by S' (adding log S' to log I') as indicated. This shifts the curves in a horizontal direction but does not alter them otherwise. All details in the interpretation of these curves will be essentially as presented by Straus and Goldstein for the non-competitive case.

The curve of competitive equilibrium in the system cholinesterase-physostigmine-acetylcholine is represented by the experimental points plotted about the curve C in Fig. 3, the data for which are found in Table II*. The effect of introducing the usual large concentrations of S is always in the direction of *reducing the zone* in which the non-competitive system might have operated. Let us assume $E_I' > 0.1$ so that the non-competitive system is in zone B; E_I'/S' will probably still be less than 0.1 (because of the magnitude of S'—64.4

* See Appendix for Tables II to VI.

in our experiments), so that the competitive system is in zone A. We have already discussed the effect of S in reducing a zone C system to zone B.

With regard to curve C of Fig. 3, it was readily apparent from the concentration of S and the approximate value of K_I, that, although the system might have been in zone B under non-competitive conditions, it *must* now be in zone

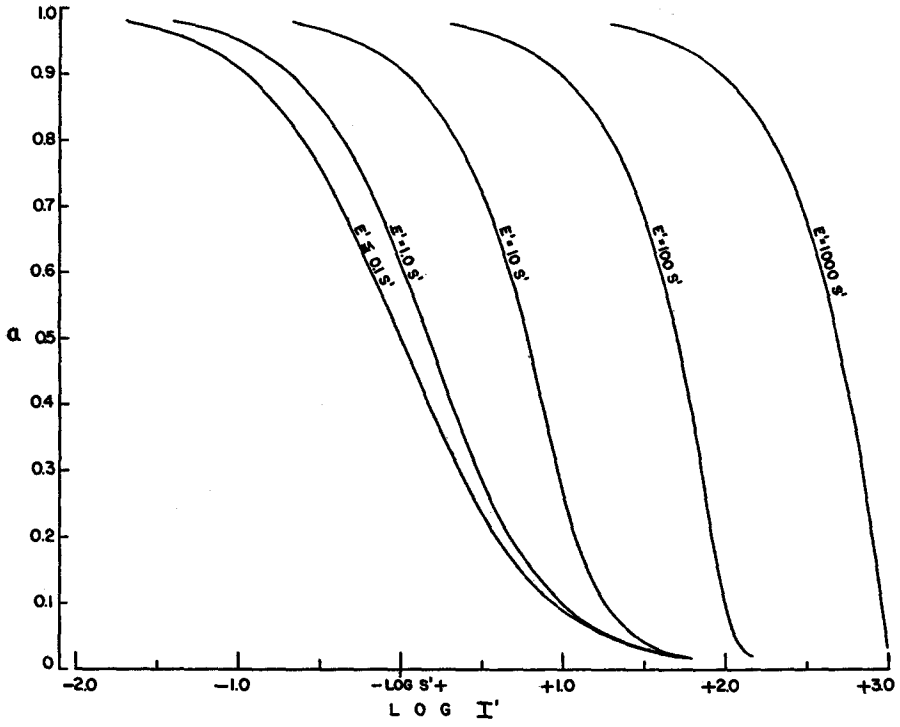

FIG. 2. Zone curves for $n = 1$. Fractional activity, a, as a function of $\log_{10} I'$ for discrete values of E_I'. These curves represent both non-competitive and competitive equilibrium. For competitive equilibrium the figures on the abscissa are added to $\log_{10}$ of the specific concentration of substrate (S') as shown. For non-competitive inhibition substitute 0 for $\log S'$ on the abscissa, and 1 for S' on the curves themselves, which will then describe the equilibrium in the absence of substrate.

A, unless E exceeded 10^{-4} molar. We therefore drew the best zone A curve for the experimental points, and the fit is seen to be quite satisfactory. Knowing S and K_S (from the experiment of Fig. 1) and reading off I' when $a = 0.5$, we can then calculate the value of K_I:

$$\frac{I}{K_I} = \frac{S}{K_S} \cdot \frac{1 - a}{a}$$

and when $a = 0.5$,

$$K_I = \frac{K_S I}{S}.$$

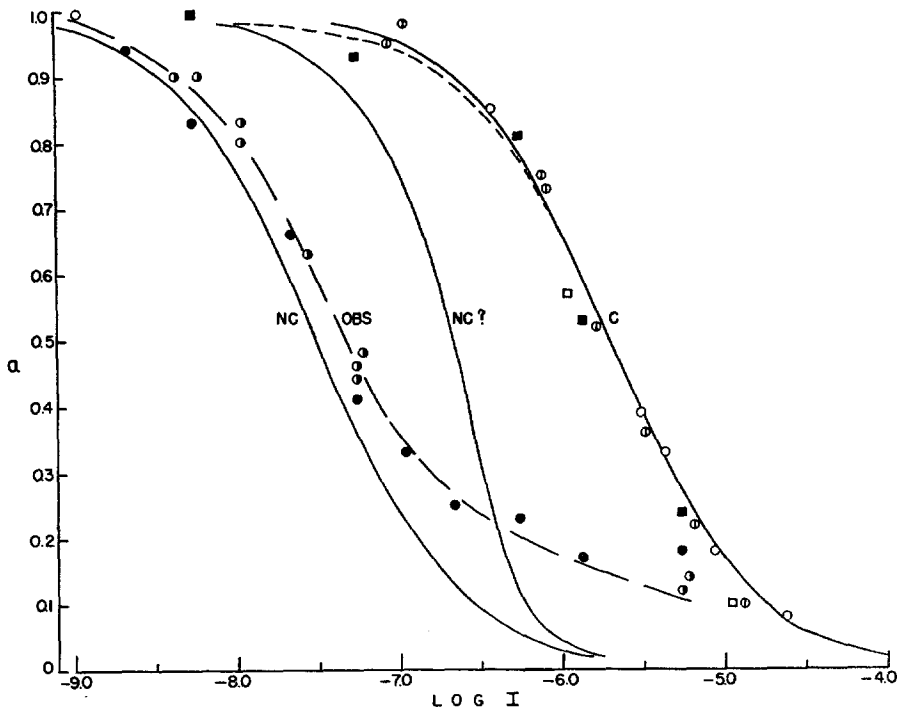

FIG. 3. Competitive equilibrium (experimental and theoretical). *For all curves:* a is plotted as a function of $\log_{10}$ of the molar physostigmine concentration in the reaction mixture.

OBS: the curve drawn through observed activities as determined 3 to 23 minutes after substrate addition. ● = duplicate determination; ◑ = single determination.

C: the zone A curve fitted to experimental points representing 20 minute readings at full equilibrium of the E-I-S system. ⦶ = duplicate determination; ○ = single determination. □ and ■ = activities approached by the curves of Figs. 8 and 9 respectively at equilibrium. The broken line of the upper part of curve C indicates the slight falling off observed at this substrate concentration when E_f is not neglected in deriving the equation.

NC: the theoretical zone A non-competitive curve for the value of K_I obtained from curve C. This curve correctly lies to the left of the 3 to 23 minute curve OBS.

NC?: the absurd non-competitive curve for the value of E and K_I obtained through application of the statistical methods described in the text, to curve C. The absurdity of this curve consists in its position far to the right of OBS.

K_I for dog serum, obtained in this way, is found to be 3.11×10^{-8} and we believe that the excellent fit of the experimental points to the curve, the independence of K_I from E in this zone, and the conditions of complete competitive equilibrium make this constant subject to little error.

As Straus and Goldstein stated, and as is obvious from equation 7 $A_I A_S$ although K can be obtained in zone A, there is no way of arriving at an estimate of E, which does not appear in the equation. The points on the curve OBS in Fig. 3 represent the data obtained from readings in the period 3 to 23 minutes after addition of substrate. Most investigators who have sought a value of E have used this type of curve and assumed that it was essentially non-competitive. Applying the non-competitive equations, none have had difficulty in arriving at a value for E. Straus and Goldstein, on the basis of just such methods, found $E = 2.7 \times 10^{-8}$ in 22.2 per cent horse serum, giving $E_I' = 0.73$, so that the system apparently acted in the lower part of zone B. It is now clear from the experiments presently to be described that the 3 to 23 minute curve is certainly not non-competitive, but represents a transitional stage in the entrance of substrate into the equilibrium. In other words, in Fig. 3, the *true* non-competitive curve relating a to log I' in the absence of substrate, lies somewhere to the left of the curve OBS, while the final competitive equilibrium is represented by curve C, far to the right. To make this clearer, let us assume a certain concentration of inhibitor (log $I = -7$) equilibrated with enzyme. When substrate is added for the 3 to 23 minute determination, a is found to be 0.35. This means that a must have been *less* than 0.35 at the moment of substrate addition. When the new competitive inhibition has been attained, with this same concentration of inhibitor the activity has risen to 0.94. This "competition effect" will be analyzed at great length subsequently; it is brought in here only to show that the common 3 to 23 minute curve is unreliable for the determination of any constant, least of all E.

Although not as satisfactory as a definitive solution would be, there is still one approach we can make to the problem of determining E. It is clear from the above discussion that every point on the true non-competitive curve (NC) must lie to the left of the curve OBS. Let us take the point $a = 0.5$ on OBS; the corresponding value of I is 4.37×10^{-8}. We may then say that:

$$I_{NC} < 4.37 \times 10^{-8} \qquad \text{when } a = 0.5.$$

Then

$$<4.37 \times 10^{-8} = 3.11 \times 10^{-8} + 0.5E \qquad \text{(Equation 2}B\text{)}$$

and

$$E < 2.52 \times 10^{-8}.$$

If we apply the same reasoning to the point $a = 0.7$, we can show that $E < 1.8 \times 10^{-8}$. By taking larger values of a, we might delimit E still further, but the scatter of the experimental points becomes too great in this range to make such a procedure reliable.

It is of some interest to note that had we applied the illegitimate statistical method discussed on page 535 to the data of curve C, we would have arrived at a definite and not negligible value of E (3.7×10^{-8}), large enough to place the system in zone B. One might easily be misled by such a result were the process of reasoning omitted, whereby it was shown that the system *must* be in zone A. Fortunately we are able further to show the impossibility of so large an E by plotting the non-competitive curve for that value. This is the curve NC? in Fig. 3 which is absurd by virtue of its position considerably to the *right* of the experimental curve of 3 to 23 minute readings (OBS).

In summary we may say that while K_I has been determined definitely, E has not. Its maximum value may be 1.8×10^{-8} (in 4.54 per cent dog serum) in which case $E_I' = 0.58$ and the non-competitive system is in the lower part of zone B; or E_I' may be less than 0.1 ($E < 3.1 \times 10^{-9}$) in which case the system is in zone A and E cannot possibly be determined from this type of experiment. The curve NC in Fig. 3 represents the non-competitive curve if $E_I' < 0.1$; the curve for $E_I' = 0.58$ would lie between NC and OBS. The competitive system is in zone A in any case, so that E cannot be determined from competitive data.

It is of interest to examine the equation 7 $A_I A_S$, which represents a common situation, and precisely the one depicted in our curve C of Fig. 3. The equation may be re-written:

$$\frac{I'}{S'} = \frac{1 - a}{a}$$

This states, as has already been pointed out, that a is a function of the ratio I'/S', which is in turn equal to the product $\frac{I}{S} \cdot \frac{K_S}{K_I}$. When $a = 0.5$, $\frac{I}{S} = \frac{K_I}{K_S}$; in other words to obtain half inhibition of the enzyme requires no absolute amount of either substrate or inhibitor, but a fixed ratio of one to the other. Each molecule of I "neutralizes" a number of S molecules equal to the ratio of their respective dissociation constants.

From a somewhat different standpoint we may rewrite the equation:

$$\frac{1 - a}{I'} = \frac{a}{S'}$$

$$\frac{\frac{EI}{E}}{I'} = \frac{\frac{ES}{E}}{S'}$$

and

$$\frac{\frac{EI}{I}}{\frac{ES}{S}} = \frac{K_S}{K_I}$$

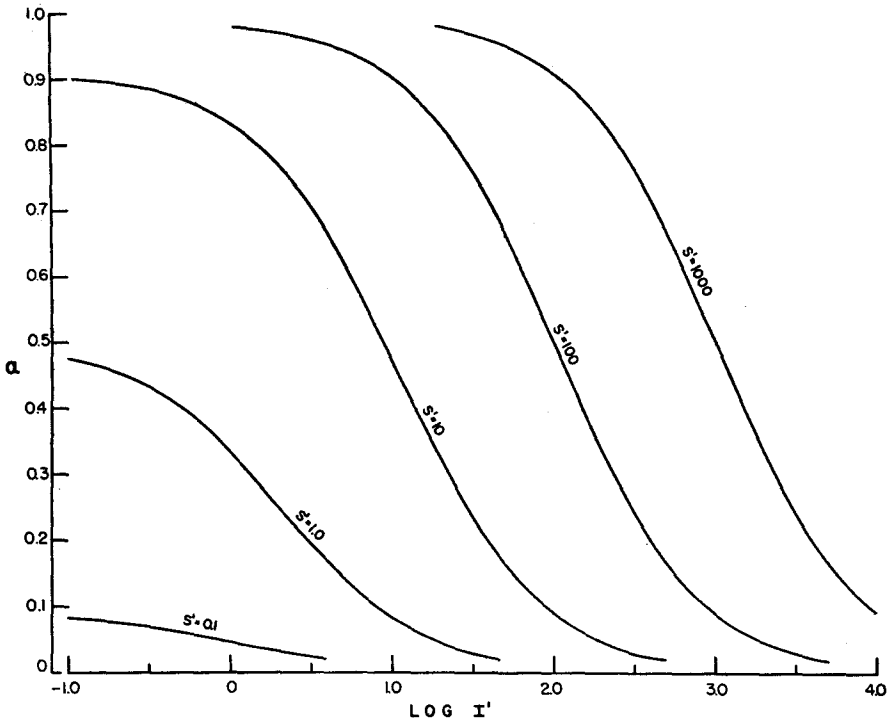

FIG. 4. Suboptimal substrate concentration. Fractional activity, a, as a function of $\log_{10} I'$ for discrete values of S'.

This states that the ratio of the combined fractions of inhibitor and enzyme respectively is equal (for all values of a) to the inverse ratio of their dissociation constants.

Suboptimal Substrate Concentration.—

Our previous discussion touched upon a few of the effects of suboptimal substrate concentration. These effects are best illustrated by Fig. 4, in which a is plotted against log I' for various discrete values of S'. To keep the discussion reasonably simple, we have assumed zone $A_I A_S$. We may not, however, simplify by neglecting E_f, for as we have seen it is precisely when S' is small that the enzyme may be unsaturated (and E_f significant) even though both S and I be present. In Fig. 4 we find that while the curves for high S' resemble

the zone A competitive curve of Fig. 2, as S' falls off, the curves become asymptotic to some lower value than $a = 1.0$. Actually, as I' approaches zero, each curve approaches that activity which would be predicted by Fig. 1 and equation $4A$ for that particular specific concentration of substrate. This is seen in the approach of equation $6A_IA_S$

$$I' = S' \cdot \frac{1 - a}{a} - 1$$

to

$$S' = \frac{a}{1 - a} \qquad \text{as } I' \to 0.$$

Thus, the activity a, in a competitive system does not vary from 0 to 1, but only from 0 to $S'/(1 + S')$ (which approaches 1 as $S' \to \infty$). The characteristic skewed shape of the curve is observable even at the high S' we have used; this can be observed in the falling off of the upper part of curve C (broken line) in Fig. 3, to approach the value $a = 0.985$.

The competitive curves of Fig. 2 are therefore wholly valid only when S' is very large, so that E_f may be neglected. These curves have characteristic slopes, which are the same in each zone as those found by Straus and Goldstein for the non-competitive curves—varying from 0.575 in zone A to 1.151 in zone C. We have already seen that when S' is suboptimal these slopes tend to fall off, a phenomenon that is only demonstrated in the full equations which include E_f. Equation 6 A_IA_S can be differentiated to give

$$-\frac{da}{d \log I'} = \frac{\left[S' \cdot \frac{1 - a}{a} - 1\right] a^2}{S'} \tag{8}$$

and when $a = 0.5$:

$$-\frac{da}{d \log I'} = 0.575 \frac{S' - 1}{S'}$$

Here when S' is very large the slope is 0.575, but it becomes progressively smaller as S' is reduced. Equation 8 shows that the falling off of slope when S' is made smaller applies to all parts of the curve but is most marked for the higher values of a.

It should now be evident that with suboptimal substrate concentrations, the uninhibited enzyme activity is no longer equal to $V_{max.}$. Consequently for any enzyme one must first determine the true $V_{max.}$ experimentally, or, knowing the constants, must calculate it on the basis of the proper equation. One must then express all observed velocities in terms of the true a. Reference to the curve $S' = 1$ in Fig. 4 shows how great would be the error of considering

as $V_{max.}$ that velocity attained by uninhibited enzyme at this substrate concentration; activity values expressed on this basis would err by about 100 per cent and would be worthless for analytical purposes.

The curves of Fig. 4 show certain characteristic features. In the region $I' > 100$, the curves follow equation 7 $A_I A_S$; it is the region where any given inhibition can be maintained by varying inhibitor and substrate together without changing their ratio. In the region $I' < 0.1$, all the curves have become practically horizontal; this means that activity is now determined only by substrate, changes in inhibitor concentration having insignificant effect.

These curves also show the effect of diluting a competitive system. The reader should consult pages 568–570 of Straus and Goldstein for the method of determining dilution effect with curves of this type. The results, expressed qualitatively, are as follows: There is no change in a on dilution when $I' > 100$. There is increasing change when $100 > I' > 0.1$; and the maximal decrease in activity on dilution (we refer, of course, to a decrease in a, not to the normally expected decrease in observed velocity) occurs in the region where $I' < 0.1$. The dilution effect is a result of the dissociation which occurs on diluting a reversibly associated complex. Here EI and ES both dissociate, but the observed decrease in fractional activity reflects the dissociation of ES alone.

A vertical segment of Fig. 4 at any point shows the variation in a with changing S' for a given value of I'. This relationship could more easily be demonstrated by plotting a against log S' for discrete values of I'.

Let us now consider the case of an enzyme which works upon a moderately small concentration of substrate *in vivo*; *e.g.*, $S' = 0.1$ (for acetylcholine bromide this is equal to 28 micrograms per cc.). Reference to Fig. 4 reveals that the maximum possible activity with this substrate concentration is about 0.10. To reduce this activity by one-half requires that I' be somewhat less than 1.0. On the other hand, if with the same enzyme *in vitro* S' were 100, allowing a maximum activity of 0.99, we should need $I' = 100$ to reduce the activity by one-half. Thus inhibitor is most potent when very little substrate is present.

Now let us suppose that an enzyme determined *in vitro* against $S' = 100$ shows an activity $a = 0.5$. Fig. 4 shows that this same concentration of inhibitor has an enormously greater effect when S' is small. For example, if $S' = 1.0$, activity will be reduced to 0.01, which is 2 per cent of the maximal rate for this substrate concentration; and if $S' < 0.1$, the activity is reduced to practically nil.[2]

These few examples demonstrate that one can hardly expect any correlation

[2] The assumption is made in this whole discussion that the *in vitro* determinations are done in undiluted serum, or that appropriate dilution corrections have been applied.

between *in vitro* data with high substrate concentration and those *in vivo* where S' may be exceedingly small. But we know so little about actual substrate concentrations *in vivo* that it does not seem profitable to explore the quantitative aspects of this question any further at this time.

The fractional activity, a, of an enzyme is a direct measure of the rate of hydrolysis of the substrate. Since

$$aE = (ES)$$

and

$$-\frac{dS}{dt} = k_D(ES)$$

it follows that

$$a = \frac{1}{k_D E}\left(-\frac{dS}{dt}\right)$$

and

$$k_D E = \left(-\frac{dS}{dt}\right)_{a=1}$$

For 4.54 per cent dog serum and acetylcholine, we find that $k_D E = 0.0635$ mM per liter reaction mixture per minute. A full treatment of the destruction of substrate by enzyme will be found on page 570 where physostigmine is considered as the substrate. We merely wish to show here that a at any time is proportional to the absolute velocity of substrate hydrolysis. We have already shown that what would be an ineffective inhibitor concentration with excess substrate can produce a marked decrease in activity when substrate concentration is small. Thus the absolute velocity of hydrolysis of a substrate like acetylcholine in the serum should be so decreased that appreciable accumulation of this substance ought to occur with doses of physostigmine barely capable of causing demonstrable inhibition *in vitro*. However, we do not know how much accumulation in serum is necessary to produce physiological effects; and further, as the serum substrate concentration increases, there is a parallel increase in its enzymatic rate of destruction, thereby antagonizing the augmented accumulation rate.

An interesting observation can be made with reference to the function of the cholinesterase, or any similar enzyme, in the serum. We know that S' in serum is likely to be very much smaller than 0.1, the value used in our previous examples. (For an acetylcholine concentration of 1:200 million, $S' < 0.0001$!) The maximum activity obtainable is consequently very much less than 0.10. The absolute rate of hydrolysis is therefore extremely small, and it might appear at first sight that practically all the enzyme present, being uncombined

with substrate, were being wasted. One must recall, however, that a represents the *relative* saturation of the enzyme; if only one-tenth of the enzyme were present, a would still have the same value (since changing E in zone A does not alter a), but the absolute velocity of hydrolysis, which was previously ak_DE, would now be only $ak_D(0.1E)$, or one-tenth its previous value. It is apparent, then, that to produce the necessary rate of hydrolysis of a minute concentration of substrate, *i.e.* to hold the circulating substrate concentration down to an extremely low level, one must have a tremendous "excess" of enzyme, most of which will not (at a given moment) even be combined with the substrate molecules.

The foregoing discussion of suboptimal substrate concentration demonstrates that there is no particular reason why an investigator should work with an "excess" of substrate. It is true that the classical enzyme-substrate-inhibitor equations omitted consideration of E_f with the result that they would describe the system only when substrate was present in high concentration. Provided the descriptive equations are adequate, and the desired range of activity is covered, one may select any substrate concentration. The chief advantage of large S' is that more even coverage of the whole range of activities can be obtained. Whatever one's choice of substrate concentration, the fact remains that the results will probably not in any event be applicable to the very different concentrations obtaining under physiological conditions.

Use of Zone Phenomena for Determining Reaction Mechanism.—

All the preceding discussion has been based upon the combination of a single molecule of substrate or inhibitor with one molecule of enzyme,

$$E + X \rightleftharpoons EX$$

Straus and Goldstein showed that it was possible to extend their analysis to systems of greater complexity, in which n molecules of a substance X combined with each molecule of enzyme (n not necessarily an integer). For the noncompetitive case, if we consider

$$E + nI \rightleftharpoons EI_n$$

and the mass law equation:

$$\frac{(E_f)(I_f)^n}{(EI_n)} = K_I$$

we can proceed, by the same steps as were used to derive equation $3B$, to the general equation:

$$I' = \sqrt[n]{\frac{1-a}{a}} + n(1-a)E_I' \qquad (9)$$

where we have defined

$$I' \equiv \frac{I}{\sqrt[n]{K_I}}, \quad \text{etc.,}$$

Similarly

$$S' = \sqrt[n]{\frac{a}{1-a}} + naE_I' \tag{10}$$

For each value of n, there will be a distinct set of "dose-effect" curves, whose characteristic slopes, shapes, zone boundaries, etc., will depend directly upon n.

Slopes can be found by differentiating a with respect to log I':

$$-\frac{da}{d\log I'} = 2.303\left[\frac{na^2}{\sqrt[n-1]{\frac{a}{1-a}} + n^2a^2E_I'}\right]\left[\sqrt[n]{\frac{1-a}{a}} + n(1-a)E_I'\right] \tag{11}$$

For the slope at the midpoint, set $a = 0.5$, so that:

$$-\frac{da}{d\log I'} = 2.303 \times \frac{0.25n(1 + 0.5nE_I')}{1 + 0.25n^2E_I'}$$

The limits of variation in slope are given by:

$$\lim\left(-\frac{da}{d\log I'}\right)_{E_I' \to 0} \qquad \text{and} \lim\left(-\frac{da}{d\log I'}\right)_{E_I' \to \infty}$$

Thus, if $n = \frac{1}{2}$, slope varies from 0.288 to 1.151;
if $n = 1$, slope varies from 0.575 to 1.151;
if $n = 2$, slope is 1.151 in all zones.

In zone A, slope is equal to $0.575\,n$. Thus, while in zone C the slope is the same for every n, in zone A the variation is so great that slope might well be used as a criterion for the determination of n, a point to which we shall later return.

From a practical standpoint n does not vary very greatly. For enzyme-substrate and enzyme-inhibitor reactions it is likely to be either 1 or 2, or possibly $\frac{1}{2}$ (meaning that one molecule of S or I combines with two molecules of E). We shall consider in some detail the case $n = 2$, for the reaction $E + 2I \rightleftharpoons EI_2$, since it has been seriously proposed as the mechanism of several enzyme-inhibitor (and enzyme-substrate) combinations.

Fig. 5 shows the "dose-effect" curves when $n = 2$, for various values of E_I', plotted according to equation 9. The general similarity between these and the curves of Fig. 2 (where $n = 1$) is evident, but we wish to stress here certain striking differences: (1) The curves appear noticeably steeper; actually it will be seen that those for the smaller values of E_I' are twice as steep as the corresponding curves of Fig. 2. (2) The curves attain a maximum spacing much

more quickly; *i.e.*, at smaller values of E_I'. (3) In the region where E_I' is great, the position of the curves on the log I' axis has shifted log n units (here 0.3) to the right.

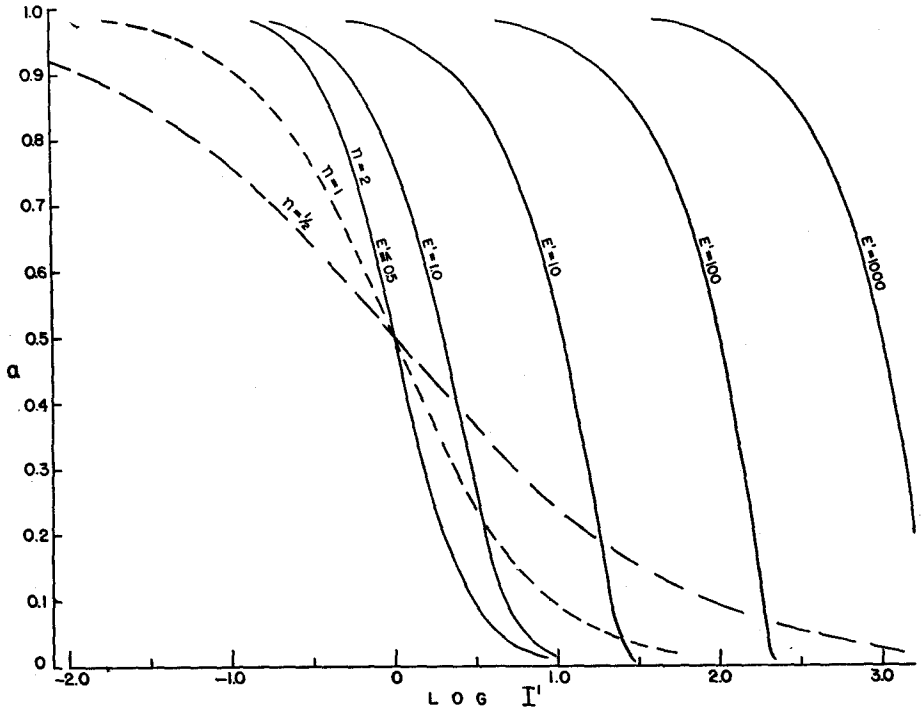

FIG. 5. Zone curves for $n = 2$. Fractional activity, a, as a function of $\log_{10} I'$ for discrete values of E_I', when $n = 2$. The zone A curves for $n = 1$ and $n = \frac{1}{2}$ are also shown for comparison. These curves are non-competitive but S' could properly be introduced exactly as in Fig. 2.

Of these three distinctive features we have already taken up the question of *slope*. The *spacing* of the curves is determined by the zone boundaries, which are shown in Fig. 6. This figure is plotted by setting the full equation

$$I' = \sqrt{\frac{1-a}{a}} + 2(1-a)E_I'$$

equal to either simplified form *with an acceptable increment of error in a*. Thus for the boundary between zones A and B:

$$\sqrt{\frac{1-a}{a}} + 2(1-a)E_I' = \sqrt{\frac{1-(a+\Delta a)}{a+\Delta a}}$$

and for the boundary between zones B and C:

$$\sqrt{\frac{1-a}{a}} + 2(1-a)E_I' = 2(1-a-\Delta a)E_I'$$

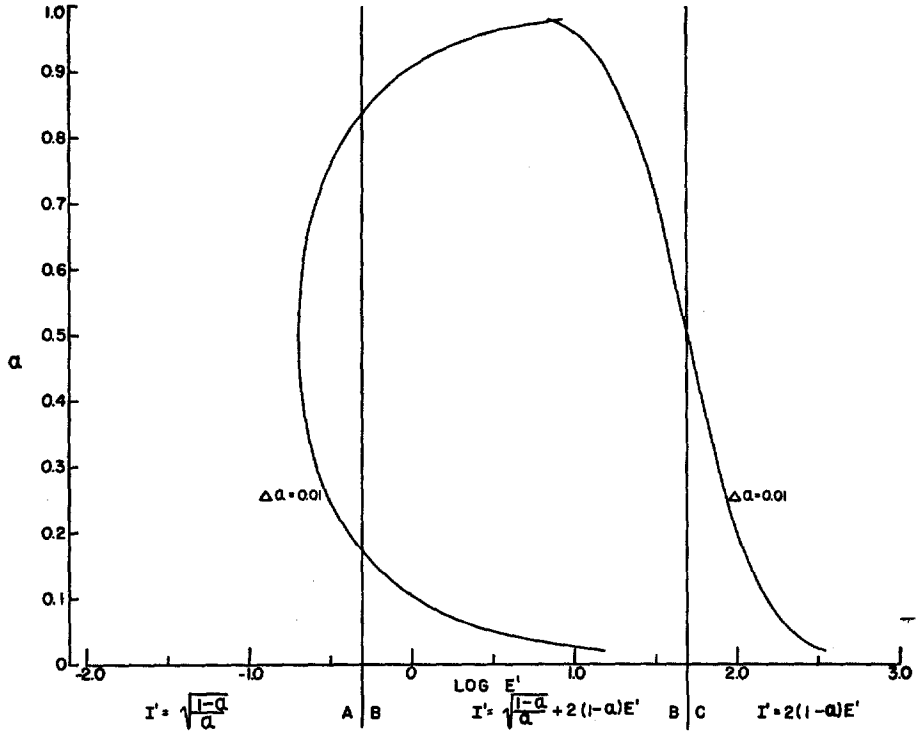

FIG. 6. Zone boundaries for $n = 2$. The two boundary curves show the exact relation between log E_I' and a for the condition that either simplification of the zone B equation be used without exceeding an error of 0.01 in the value of a. The vertical lines are practical zone boundaries arbitrarily adopted, neglecting the variation in a. To the left lies zone A; in the center zone B; to the right zone C.

The acceptable error Δa, is chosen to be 0.01. Fig. 6 shows the values of E_I' for which the simplified forms may be used so that Δa at any given a will not exceed 0.01. For practical use, we settle upon a rough average E_I' for each zone boundary: $E_I' < 0.5$ and $E_I' > 50$ for the boundaries AB and BC respectively; these boundaries should be satisfactory, provided reasonable values of a are not exceeded. Comparison with the zone boundaries in the case $n = 1$ allows one to conclude that as n is increased, zone B becomes narrower, while zones A and C both expand. Consequently a system which would operate

in the lowest part of zone B if n were equal to 1 would lie in zone A if n were 2; and in similar fashion a system thought to lie at the upper part of zone B would really be in zone C if n were 2.[3]

The shift in the *position of the curves* to the right, if n is increased, and to the left if n is decreased, is first noticeable in zone B, and becomes maximal in zone C, where it is equal to log n. That there is no such shift in zone A is shown by the two zone A curves in Fig. 5 (broken lines) for $n = 1$ and $n = \frac{1}{2}$, whose mid-points are identical with that of the curve for $n = 2$.

What does this tell us about the relative potency of inhibitor or substrate operating according to the mechanism $n = 1$, or $n = 2$? It reveals that in zones B and C, inhibitor is less effective when n is greater, a higher concentration of I being required to produce half-inhibition. Since it seems likely that an enzyme such as cholinesterase (which is known to be highly concentrated at the synaptic regions) operates in zone C *in vivo*, we must modify Eadie's (5) conclusion that it is "possible to attain practically complete inhibition much more readily" since "the number of enzyme molecules blocked increases with the square of the inhibitor concentration, rather than with the first power." The decrease in inhibitor potency as n is increased in zone C is a striking example of a conclusion which may have physiological significance and which could not have been derived from classical zone A principles. Furthermore, even in zone A there is not any absolute increase in inhibitor potency when $n = 2$, and reference to Fig. 5 shows that in this zone, while a given inhibition can be obtained with less I' for activities below 0.5, the reverse is actually true when a is greater than 0.5. The only "increase in potency" consists in the fact, reflected by the doubled slope, that the entire range of inhibition can be covered by a relatively small *change* in I', and it is only in this sense that Eadie's statement is correct.

The equations for competitive inhibition for any n can be derived by combining the methods employed in the derivation of equations 6 and 9, yielding:

$$I' = \sqrt[n]{(S' - n a E_S')^n \cdot \frac{1-a}{a} - 1} + n\left[1 - a\left(1 + \frac{1}{(S' - n a E_S')^n}\right)\right] E_I' \qquad (12)$$

All the usual simplifications and approximations can be applied to this equation and the resulting expressions will give curves bearing the same relation

[3] The actual values of E_I' for different values of n are entirely comparable. This is because on the one hand we define $E_I' \equiv E/\sqrt[n]{K_I}$, while on the other, our determination of K_I is based on the concentration of I when $a = 0.5$, in the zone A form of equation 9, where $K_I = I^n$. As a result the numerical value of E_I' is the same for a given molar E, regardless of the value attributed to n.

to the non-competitive ones as we have already demonstrated in detail for the case $n = 1$. The slope of the competitive curve for $n = 2$ can be shown to be:

$$-\frac{da}{d \log I'} = \frac{\sqrt{S' \cdot \frac{1-a}{a} - 1} + 2\left[(1-a) - \frac{a}{S'}\right] E_I'}{\frac{S'}{2a^2\sqrt{S' \cdot \frac{1-a}{a} - 1}} + 2E_I'\left(1 + \frac{1}{S'}\right)} \tag{13}$$

and when $a = 0.5$, this reduces to:

$$-\frac{da}{d \log I'} = \frac{\sqrt{S' - 1} + E_I'\left[1 - \frac{1}{S'}\right]}{\frac{S'}{0.5\sqrt{S' - 1}} + 2E_I'\left(1 + \frac{1}{S'}\right)}$$

which yields the familiar slope of 1.151 in all zones when S' is sufficiently large. We have assumed here that the *substrate* combines with enzyme according to a 1:1 mechanism, but the result would be the same regardless of the value of n in the substrate-enzyme combination.

Straus and Goldstein pointed out that the dilution effect might be expected to change significantly if the value of n were altered, and they presented a general equation for dilution, in zone A, which can be modified to:

$$a_2 = \frac{a_1}{1 - (N^n + 1)(1 - a_1)} \tag{14}$$

where N is the factor of dilution,

a_1 is the original activity at concentration E_1

a_2 is the activity at concentration NE_1

The equation for dilution in zone B, which would correspond with their equation $7B$, involves solution of a cubic which we believe is too complicated to be of any real use. Practical dilution curves for zone B can be constructed, however, from the "dose-effect" curves for various desired values of E_I' (*e.g.*, those in Fig. 5). This has been done for the case $E_I' = 0.73$ in 22.2 per cent serum, to correspond with the dilution effect found by Straus and Goldstein, assuming n to be 1. The results at this value of E_I' are so little different from those in zone A that we need not be concerned here with these authors' probable overestimation of E_I', which has already been thoroughly discussed. Fig. 7 shows the effect of diluting such a system, where $n = 2$; it is read and interpreted in the same way as Straus and Goldstein's Fig. 3, with which it should be compared. The chief conclusion to be drawn from such a comparison, and one that (for zone A) could have been predicted from equation 14, is that the dissociation which occurs on dilution is more marked the greater is the value of n. *The increase of activity on diluting an enzyme-inhibitor mixture is thus considerably greater when $n = 2$ than when $n = 1$.*

We have gone into considerable detail in analyzing the general effect of variation in n, and the specific comparison of the case $n = 2$ with $n = 1$ to provide a firm basis for the following discussion of the use of zone phenomena in determining the mechanism of an enzyme-inhibitor or enzyme-substrate combination.

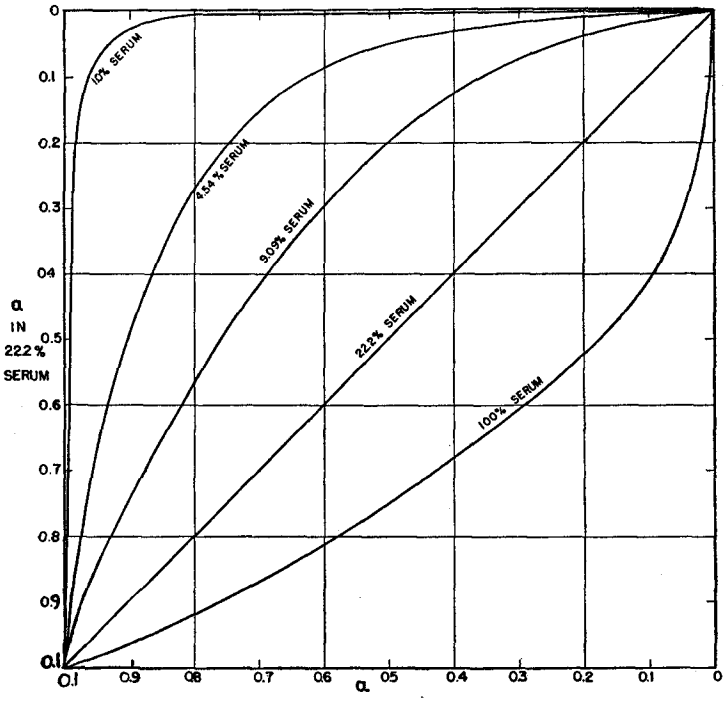

FIG. 7. Dilution effect for $n = 2$. Ordinate: fractional activity in 22.2 per cent serum. Abscissa: fractional activity at any serum concentration. Dilution or concentration of an enzyme-inhibitor mixture is represented by travelling horizontally from the original to the final serum concentration and reading off the initial and final activities from the abscissa. The ordinate was used in constructing the figure and is convenient if one is interested in activity in 22.2 per cent serum, but it is not essential in using the figure. This figure is constructed for $E_I' = 0.73$ in 22.2 per cent serum, to correspond with Fig. 3 of Straus and Goldstein.

There appears to be a widespread opinion to the effect that since (1) pharmacological experimentation usually yields valid data only in the middle portion of a range of observed effects and not at the extremes, and since (2) many different curves representing entirely different functions relating the two variables may be roughly alike in their middle portions, and since (3) the precision of the pharmacologist's observations is not comparable to that obtained by the physical chemist, one should hesitate to draw conclusions about the mechanism of a

reaction from observed data. We can see little merit in this reasoning for, while the first point may be generally true, the second need not be, while the third may not even constitute a handicap provided one knows the error to which one's data are subject.

The most direct criterion of the value of n is the slope of the "dose-effect" plot, and this slope is the same regardless of the units used to express inhibitor concentration. Here the differences in slope are so great that confusion is hardly possible unless the experimental points are unusually scattered. That the points through which the curve of Fig. 1 is drawn could not conceivably lie on a curve with double or half the slope is hardly open to doubt. Thus the actual slope, 0.575, allows one confidently to assign the value $n = 1$ to the enzyme-substrate combination in this case. We have shown that the slopes of the competitive equilibrium curves have the same significance as those of the non-competitive curves. We may therefore apply the same reasoning to the points through which curve C of Fig. 3 is drawn, and assign $n = 1$ to this reaction between enzyme and inhibitor in the presence of substrate. We should therefore conclude from these two experiments (unless there is some systematic error in the data) that *one molecule of acetylcholine or of physostigmine combines reversibly with a single molecule of cholinesterase.*

Eadie (5) studied the mechanism of combination of cholinesterase and physostigmine and concluded that in this reaction $n = 2$. We have just shown that on the basis of our studies on this same system such a mechanism is almost out of the question. But since we ruled out such a mechanism solely on the basis of slope, we shall now proceed to show that the observed effect of dilution is also incompatible with it. Straus and Goldstein showed that their experimental results on dilution of a dog serum-physostigmine mixture agreed quite well with the expected results calculated according to the dilution equation for $n = 1$. In Table I we have entered the experimental data of these authors (from their Table III) together with the values that would be expected for each concentration, if $n = 2$, according to Fig. 7. The discrepancies are not only excessively great, but follow an unmistakable trend rather than a random scatter, indicating the type of systematic error in calculated values which would result from use of an equation that did not correctly describe the reaction mechanism.

On the other hand there is no doubt that Eadie's conclusion follows from his data, which are in themselves consistent. Thus, if the data of his Fig. 4 are replotted according to our method, a curve with slope 1.15 is obtained, showing that $n = 2$ (in zone A). Therefore the problem of reconciling Eadie's results with our own comes down to showing, if possible, some systematic error in his data which could account for the discrepancy. His method involves the addition of enzyme to a substrate-inhibitor mixture followed by titration of released acetic acid against 0.01 N NaOH for 20 minutes. We have found that

under similar conditions the combination of physostigmine with enzyme is rather slow and that the hydrolytic rate in the first 20 minutes is significantly higher than at equilibrium, and this discrepancy is greatest for concentrations

TABLE I

Effect of Dilution on Activity, a, if n = 2

Serum concentration in per cent of undiluted serum	1.00	4.54	9.00	22.2	100
I/E					
1.1	**0.980**	0.725	0.470	0.175	0.015
	0.995	**0.930**	0.815	0.590	0.280
	0.995	0.965	**0.900**	0.745	0.490
	0.995	0.960	0.875	**0.700**	0.425
1.8		**0.91**		0.525	
		0.96		**0.700**	
2.1	**0.98**	0.725	0.47	0.175	0.015
	0.99	**0.82**	0.61	0.305	0.050
	0.99	0.88	**0.72**	0.445	0.125
	0.99	0.91	0.77	**0.52**	0.195
9.1		**0.64**		0.11	
		0.82		**0.31**	
10.7	**0.81**	0.125	0.025	0.005	~0
	0.92	**0.46**	0.20	0.04	~0
	0.955	0.59	**0.32**	0.08	0.005
	0.98	0.70	0.44	**0.15**	0.01
21.5	**0.78**	0.12	0.08	0.005	~0
	0.87	**0.28**	0.10	0.02	~0
	0.925	0.47	**0.21**	0.04	~0
	0.965	0.63	0.36	**0.10**	0.005
215	**0.24**	0.02	~0	~0	~0
	0.84	**0.14**	0.04	0.005	~0
	0.875	0.30	**0.10**	0.02	~0
	0.92	0.46	0.20	**0.04**	~0

of inhibitor producing moderate inhibitions. Since Eadie uses an equation for competitive equilibrium while such equilibrium (at least according to our experiments) does not really exist throughout the 20 minutes of his determination, the points of his Fig. 4 are warped downward precisely in the middle of his range of data, producing an apparent systematic divergence from linearity. The correspondence of the points to the curve for $n = 2$ would then be for-

tuitous. Our results, on the other hand, were obtained from readings in an *E-S-I* mixture at full equilibrium, and should not be subject to this error. However, it must be admitted that even the data of Straus and Goldstein, or our curve OBS of Fig. 3, which are obtained from readings made 3 to 23 minutes after substrate addition, agree with the mechanism $n = 1$ and are incompatible in a number of respects with $n = 2$.

One may wonder how far it is legitimate to apply these concepts to physiological phenomena. If a physiological effect is measured and a graded response obtained as a function of varying concentration of drug, can the slope of this function (when plotted as $da/d \log I$) be accorded the same significance as the slopes we have been discussing? This is difficult to decide if we are ignorant of the intermediary steps by which the effect is mediated and may not even know what enzyme is involved, or, for that matter, that enzyme inhibition is the responsible factor. Nevertheless it is interesting that one can demonstrate a variety of different types of reaction, both *in vitro* and *in vivo*, which give curves like those of Fig. 2 or Fig. 5 and whose slopes fall near the significant values 0.288, 0.575, or 1.151. Such similarities are usually obscured by the variety of methods used in plotting the data, but can be brought out by replotting according to the procedures we have outlined.

Such examples can be cited from the review of Clark (1), who not only presented such material in "dose-effect" curves of the type we have described, but also recognized the fact that the shape of these curves reflected the value of n. Thus the action of mercuric nitrate upon urease (data of Jacoby) is seen to be represented by a curve with slope 0.575 at the midpoint so that $n = 1$. The inhibition of blood catalase by silver nitrate (data of Bleyer) yields a slope 0.288 and therefore it would appear that $n = \frac{1}{2}$. Potassium cyanide acts upon peptidase (data of Linderstrøm-Lang) to give a curve with slope 1.151 so that $n = 2$.[4]

Interesting *in vivo* examples can be selected at random from the literature. Dubos and coworkers (8) found that a graded inhibition of the oxygen uptake of *S. aureus* cultures could be produced by varying concentrations of tyrocidine. When the data of their Table I (for 120 to 150 minutes after inhibitor addition) are plotted with a as ordinate (letting a equal per cent of normal $Q_{O_2} \times 1/100$) and log concentration of tyrocidine as abscissa, a sigmoid curve is obtained whose slope is close to 0.575. This might be interpreted to mean that one molecule of tyrocidine combines reversibly with one molecule of the enzyme whose inhibition is the key factor in blocking respiration.

In a very different type of system Astwood and Bissell (9) have shown that varying concentration of thiouracil (which is presumed to inhibit an enzyme

[4] Clark incorrectly concludes from this curve that $n = \frac{1}{2}$ so that one molecule of cyanide combines with two of peptidase, when actually the reverse is the case.

responsible for some phase of thyroxin synthesis) in the drinking water of rats produces a graded effect upon the iodine content of the thyroid. When the data of their Fig. 3 are plotted in the proper way (letting $a = 1$ when thyroid iodine is 77.5 mg. per cent, in the absence of inhibitor), a sigmoid curve of slope 0.575 is obtained. This may then indicate a 1:1 combination of thiouracil with the particular enzyme concerned. From the midpoint of this curve we may also obtain a value of K_I, which, although purely empirical, may still be quite useful. This constant is affected by such factors as the amount of inert protein binding inhibitor molecules, and the loss of inhibitor into tissues where it can have no effect. But as an empirical constant for given physiological conditions it still is a convenient comparative way of expressing inhibitor potency.

Of course in a complex reaction, the slope of the "dose-effect" curve may reflect only the mechanism of the *limiting stage* of the reaction, and not necessarily that of the crucial enzyme-inhibiting stage.

A word is in order here about the extent to which inhibition of an enzyme in serum reflects the inhibition of the same enzyme in tissues. It will be recalled from equation 2 that under all circumstances, in zones A, B, and C, the concentration of *free inhibitor*,

$$I_f = K_I \cdot \frac{1 - a}{a}$$

This states that the activity, a, of the enzyme is always determined by free inhibitor, for a given K_I. This seems at first to be contrary to the obvious fact that inhibition depends upon *combined* inhibitor. But if one imagines that a certain "pressure" of free inhibitor is always required to maintain a certain saturation of the enzyme, the contradiction will be resolved. If the inhibitor be a relatively small molecule, it will be clear that while E and EI are not diffusible, I_f should freely pass semipermeable membranes, so that *its* concentration ought to be the same within and without the vascular compartment, at equilibrium.[5] And if free inhibitor in serum $\left(K_I \cdot \frac{1 - a_s}{a_s}\right)$ equals free inhibitor in tissues $\left(K_I \cdot \frac{1 - a_t}{a_t}\right)$, it follows that $a_s = a_t$. In other words, the enzyme inhibition is always the same in tissue as in serum, regardless of the zone and of the enzyme concentration in either, provided only that adequate time is left for diffusion, so that a true equilibrium is achieved. In the case of an enzyme which is present only in the tissues, measurement of the free

[5] Davis (10) has verified this assumption for protein-bound and free sulfonamide equilibrated across a semipermeable membrane. The Donnan effect can probably be neglected.

inhibitor in the blood at equilibrium is sufficient for determining the degree of inhibition of the enzyme, for the same reasons. In either type of experiment diffusion as a significant factor can probably be overcome only with a continuous infusion technique; single doses of drug should produce a lag in inhibition in the tissues as compared with the circulating serum, and because of simultaneous excretion, destruction, etc., the expected level of inhibition may never be reached.

Several investigators (11, 12) have confirmed the fact that cholinesterase is not dialyzable, and that inhibition by physostigmine is reversible by dialysis. Preliminary experiments by the author appear to confirm the prediction that fractional activity of different concentrations of cholinesterase in the presence of physostigmine on two sides of a dialysis bag is the same when equilibrium is attained. Substitution of the red corpuscle for the dialysis bag with simultaneous determination of inhibition of serum and corpuscular cholinesterase would seem to be the next step along this line of experimentation.

We are fully aware of the hazards of attempting interpretation of the mechanism of reactions of whose very nature we are still ignorant. We wish, therefore, to emphasize the speculative character of the preceding discussions, whose conclusions lack as yet any direct experimental support.

Kinetic Studies

Experimental Method.—

The standard method employed for the experiments reported in this paper was as follows: Sterile dog serum, stored at 6°C. (there is no decrease in activity after months at this temperature) was used at a concentration of 4.54 per cent in the final reaction mixture. Substrate was 0.0805 M (S' = 64.4) acetylcholine bromide in the final reaction mixture, desiccator stored and freshly prepared for each experiment. Inhibitor was physostigmine salicylate prepared fresh to the desired concentration, which is always expressed as mols per liter reaction mixture. The method of determination was that of Ammon (13), using the Warburg constant volume manometer to measure release of CO_2 from a bicarbonate-Ringer solution medium buffered to pH 7.4. All determinations were made at 38°C. Readings were for the period 3 to 23 minutes after addition of 0.2 cc. substrate solution to 2.0 cc. containing enzyme with or without inhibitor. Appropriate correction for non-enzymatic hydrolysis was always made.

This method was altered as indicated in special experiments. Thus in the acetylcholine activity curve (Fig. 1 and Table II), the substrate concentration was varied as required. In curve C of Fig. 3 (Table III) the readings were 60 to 80 minutes after addition of an inhibitor-substrate mixture to the enzyme solution. In the experiments to follow, modifications of this standard technique will be described wherever they have been necessary.

Competitive Displacement of Inhibitor or Substrate.—

There are three ways to prove that inhibition is competitive. (1) The indirect method involves demonstrating in a plot of $1/v$ against $1/S$ that addition of inhibitor causes a change of slope but not of the intercept on the $1/v$ axis. This intercept is equal to $1/V_{max.}$ and while $V_{max.}$ is reduced in non-competitive inhibition, it remains unchanged in the competitive type (Lineweaver and Burk (14)). (2) A more direct way of showing that inhibition is competitive is to prove that with substrate present a great deal more inhibitor is necessary to produce a given effect than in its absence. This we have already shown in Fig. 3, where the differences in inhibitor requirement between curve C (which is competitive) and curve OBS (which is nearly non-competitive) are quite striking in their magnitude. If one first produces half-inhibition in the determination made 3 to 23 minutes after adding substrate, then nearly 100 times as much inhibitor is required to reproduce this same inhibition in the 60 to 80 minute reading, when competitive equilibrium has been attained. (3) The most direct method of all, possible only with a technique that permits of continuous readings during the course of a reaction, is to follow the actual time course of entrance of substrate or inhibitor (as the case may be) into the reaction. The experiments about to be described are based on this principle.

In the first type of experiment (*A*, below) we study the entrance of inhibitor into an enzyme-substrate combination, with displacement of some of the combined substrate so that a shows a progressive fall. Serum and acetylcholine are first allowed to react for 30 minutes, during which a constant hydrolytic rate is attained, and then inhibitor, in varying concentration, is added. Readings are taken every 20 minutes until equilibrium is attained, so that the variation of a with time can be directly plotted.

In the second type of experiment (*B*, below) equilibrium between enzyme and inhibitor is first attained (incubation for 1 hour). Substrate is then added and readings taken at 3 minutes and every 20 minutes thereafter, to equilibrium. Here we follow the displacement of combined inhibitor by substrate and a consequently shows a progressive rise.

It was found in these experiments that a could not be calculated simply as the ratio of observed velocity to the *initial* velocity of uninhibited serum; nor could it be based upon the velocity of uninhibited serum at *equivalent time* as the reaction progressed. For under the conditions of the experiment there was a consistent decrease in the velocity of substrate hydrolysis in normal uninhibited serum with time. This decrease could even be observed between the first and second 20 minute periods, and became more marked as hydrolysis proceeded, until the reaction stopped entirely when about 1000 c. mm. CO_2 had been released (corresponding with exhaustion of the bicarbonate buffer). One could only correlate this decreasing velocity, barely perceptible though it was at the outset, with accumulation of hydrolytic products; whether the

immediate cause was the increase in choline concentration or a shift in pH was immaterial. It was a fact that the maximal activity obtainable from the enzyme always depended upon the total CO_2 that had been released at the time. Therefore inhibited rates were always compared with the normal rate *at the same total hydrolysis*, the ratio of these two velocities being designated as a.

The time course of displacement will be shown to depend upon the absolute velocity constants for the combination of enzyme with inhibitor or substrate. The measurement of competitive displacement of one molecular species by another to determine a velocity constant, while unusual, is not without precedent. Francis and coworkers (15), in 1925, devised a similar experiment for determining the velocity constants of the rapid reactions between bromine and certain phenolic compounds. The uniqueness of the method lies in the fact that while the velocity of combination might be too great to measure directly, the presence of a reversibly combined complex enormously reduces the *immediately effective* concentration of the enzyme (E_f) without changing its ultimately effective total concentration. The resulting initial velocity of combination with the new reactant is so markedly diminished as to render it measurable by our relatively crude experimental methods.

A. Displacement of S by I.—

Fig. 8 shows the progressive decrease in a after adding two different concentrations of inhibitor to an equilibrated enzyme-substrate system. Activity in each 20 minute period is plotted at the midpoint of the period. Since the points on a given curve represent consecutive readings in the same reaction mixture, it should not be unusual for a sizable error during one period to be compensated in the next (such a situation might arise from small absolute errors in the thermobarometer readings); the tendency of the points to seesaw is therefore not to be regarded as a serious experimental error.

The rate of displacement of S by I will depend upon the rate of combination of I with the enzyme, given by

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI$$

and also by the rate of dissociation of the complex ES, given by

$$E + S \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ES$$

where k_1, k_3, and k_2, k_4 are, respectively, the velocity constants for the forward and reverse reactions. Then,

$$\frac{d(ES)}{dt} = k_3(E_f)(S_f) - k_4(ES)$$

$$\frac{d(EI)}{dt} = k_1(E_f)(I_f) - k_2(EI)$$

Substituting for (E_f) in the first equation its value in the second, we obtain:

$$\frac{d(ES)}{dt} = \frac{k_3(S_f)}{k_1(I_f)}\left[\frac{d(EI)}{dt} + k_2(EI)\right] - k_4(ES)$$

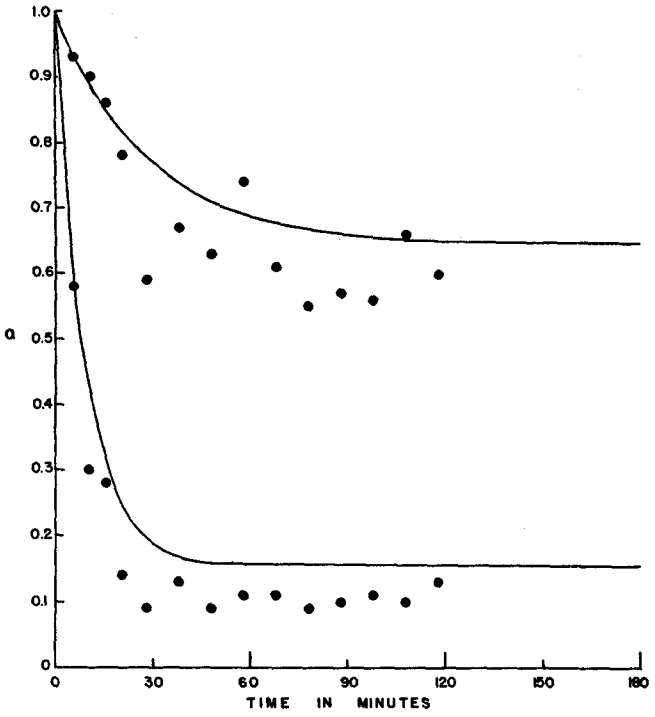

FIG. 8. Displacement of substrate by inhibitor. Physostigmine salicylate added at zero time to an equilibrated mixture of dog serum and acetylcholine. ● = observed activity for successive 20 minute periods with 1.1×10^{-6} and 1.1×10^{-5} M physostigmine, plotted at the midpoint of each period. *Solid curves*: theoretical course of the reaction, for these two concentrations of physostigmine, for $k_2 = 0.026$, and $k_4 = 0.32$.

We then assume (1) that we are in zone A with respect to substrate, so that practically $S_f \doteq S$, and (2) that free enzyme is negligible, so that $EI = (1 - a)E$; both these assumptions have been shown to be legitimate for the conditions of these experiments. Then, making the usual substitutions, with the above simplifications:[6]

$$\frac{da}{dt} = \frac{S'(1 - a) - (I_f)'a}{\frac{(I_f)'}{k_4} + \frac{S'}{k_2}} \tag{15}$$

[6] It will be recalled that $k_2/k_1 = K_I$ and $k_4/k_3 = K_S$.

Knowing that $E < 1.8 \times 10^{-8}$ (page 544), we may neglect it by comparison with the lowest (I) used in this experiment (1.1×10^{-6} M), and we may assume that practically all I is free and write the zone A equation:

$$\frac{da}{dt} = \frac{S'(1 - a) - I'a}{\frac{I'}{k_4} + \frac{S'}{k_2}} \tag{15A}$$

We can then substitute values of da/dt from the experimental curves at various values of a, to arrive by simultaneous solution at the values of k_2 and k_4.

Integration of equation 15A and setting $a = 1$ when $t = 0$, gives

$$a = \frac{1}{S' + I'} \left\{ S' + I' e^{-\frac{(S'+I')t}{\frac{I'}{k_4} + \frac{S'}{k_2}}} \right\} \tag{16A}$$ [7]

This equation can be used to construct theoretical curves giving a as a function of k_2, k_4, and t. The time scale can then be adjusted to give the best fit to the experimental points, this choice of scale automatically giving the best values of the velocity constants.

It is of interest that if the rigid equation 15 be rewritten for $a = 1$, to describe the *initial* rate of change of a, this change is seen to be entirely independent of the enzyme concentration; since at the start of the reaction all inhibitor is free and no assumptions are necessary:

$$\left(\frac{da}{dt}\right)_{a=1} = -\frac{I'a}{\frac{I'}{k_4} + \frac{S'}{k_2}}$$

Using any of these three methods, we find that $k_2 = 0.026$ minutes^{-1} so that $k_1 = k_2/K_I = 8.3 \times 10^5$ liters·mols^{-1}·minutes^{-1}; and $k_4 = 0.32$ minutes^{-1} so that $k_3 = k_4/K_S = 260$ liters·mols^{-1}·minutes^{-1}.

The solid lines of Fig. 8 are the theoretical curves drawn according to equation 16A, with the above velocity constants; their fit to the experimental points is considered to be satisfactory.

B. Displacement of I by S.—

The points of Fig. 9 show the progressive increase in a after substrate is added to an equilibrated mixture of E and I. The validity of the equilibrium competitive curve (C, Fig. 3) is strengthened by the fact that the same equilibrium is attained with a given I and S, regardless of the order of addition of reactants. The points on which that curve is based were obtained by preliminary equilibration of E, S, and I. The two black squares in Fig. 3 represent the ultimate equilibria attained by the curves of Fig. 8; the five white squares represent the

[7] As $t \to \infty$, $a \to \frac{S'}{I' + S'}$, which is the zone A value of a at competitive equilibrium.

equilibria attained by the curves of Fig. 9. It was also found that in the experiments of Fig. 9, the same equilibrium was approached if substrate was added an hour after the usual time; this was taken to mean that the presence

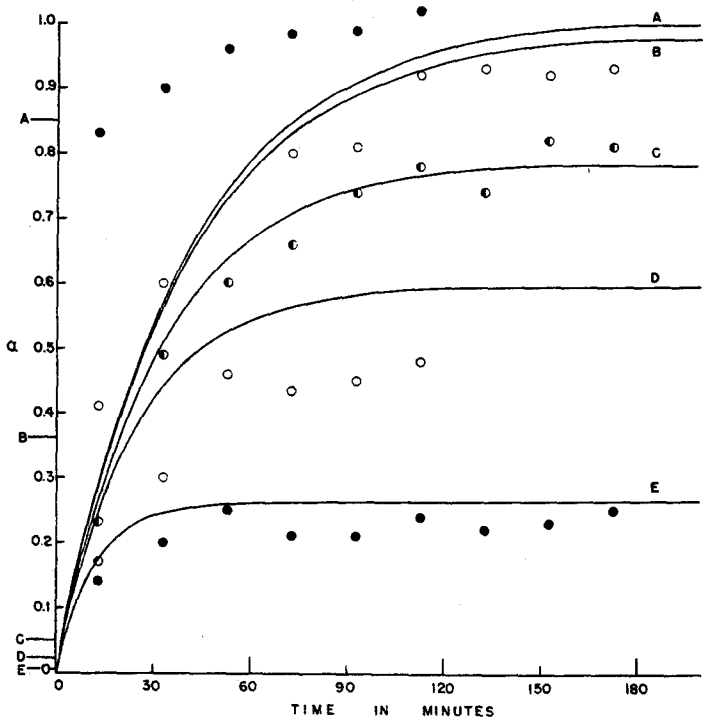

FIG. 9. Displacement of inhibitor by substrate. Acetylcholine added at zero time to an equilibrated mixture of dog serum and physostigmine. ● and ○ = observed activities for successive 20 minute periods, for each of the five physostigmine concentrations represented by the solid curves A to D, plotted at the midpoint of each period. *Solid curves*: theoretical course of the reaction, for $k_2 = 0.026$ and for $k_4 = 0.32$, for each of the following concentrations of physostigmine: A—5.5×10^{-9} M; B—5.5×10^{-8} M; C—5.5×10^{-7} M; D—1.35×10^{-6} M; E—5.5×10^{-6} M. On the ordinal axis are indicated by the appropriate letters the value of the E_I/E ratio for each physostigmine concentration before addition of substrate.

of inhibitor for a longer time did not in itself cause any change in the potential activity of the enzyme. The increase in reaction rate during the second 20 minute period as compared with the first when an inhibitor is present is in marked contradistinction to the normal slight decrease in rate of the uninhibited enzyme. This phenomenon is so reliable that it can be used as a criterion for the presence of a reversible inhibitor in an unknown solution of enzyme.

The displacement of combined inhibitor by added substrate is represented by the same equations already derived for the displacement of substrate by inhibitor, namely equations 15 and 15*A*, which describe the rate of change of a in terms of both velocity constants, without reference to the order of addition of reactants. For the initial rate, a is set equal to zero in equation 15*A*, yielding, for zone *A*:

$$\left(\frac{da}{dt}\right)_{a=0} = \frac{S'}{\frac{I'}{k_4} + \frac{S'}{k_2}}$$

To derive the theoretical curves describing the course of the reaction, equation 15*A* must be integrated after setting $a = 0$ when $t = 0$, giving

$$a = \frac{1}{S' + I'}\left\{S' - S'e^{-\frac{(S'+I')t}{\frac{I'}{k_4}+\frac{S'}{k_2}}}\right\} \qquad (17A)$$

The solid lines of Fig. 9 are the theoretical curves for five concentrations of inhibitor, plotted according to this equation, with the velocity constants previously derived. While the fit of these curves to the experimental points is not remarkably good, we believe that the deviation from the lower four curves can be explained on the basis of experimental error; our analytic method has, after all, presupposed data more accurate than our relatively crude technique could furnish. While the lower curves are consistent with the observed course of the reaction, this is certainly not true for curve A, representing the smallest inhibitor concentration. We are unable to explain this discrepancy except to point out that it does *not* arise from the assumption of zone *A* in a system which may really be in zone *B*. For while the use of I' instead of $(I_f)'$ may result in a false high value for this term, it is already (at this inhibitor concentration) so small as to be negligible in the wholly additive relationships in which it is found (*cf.* equation 17*A*); thus large differences in the value of I' at this concentration are without effect on the equation as a whole.

Whether the experimental points for curve A are at fault, or whether (as seems more likely) our treatment does not correctly describe the reaction at low inhibitor concentrations, the practical consequence of this discrepancy is equally unfortunate. For we should like to be able, on a theoretical basis, to correct any 3 to 23 minute reading to its true competitive value, from which the non-competitive EI/E ratio could be calculated. Without an acceptable equation which will fit the whole range of data, we must limit ourselves to empirical corrections. (For a full discussion of this and other corrections which must be applied to experimental observations, see Krayer, Goldstein, and Plachte (16), Discussion.)

On the ordinal axis of Fig. 9 we have indicated for each curve the non-com-

petitive value of E_f/E before addition of substrate. It might be thought that each curve would begin at that point on the a axis corresponding to its E_f fraction at zero time. This is not actually the case, for at zero time no ES is present and a must therefore be zero regardless of the amount of E_f or EI in the system. For this reason, which is well illustrated by the curves, extrapolation of the experimental points back to zero time is not a valid method of determining the initial non-competitive inhibition.

Reactions Depending on the Velocity Constants.—

The course of every conceivable reaction between two reactants at a given temperature is determined by their characteristic velocity constants. Thus, knowing k_1 and k_2 for the cholinesterase-physostigmine system, we are in a position to predict the time course of both the dilution effect and the simple combination which occurs when one reactant is added to the other.

A. Kinetics of the Dilution Effect.—

Straus and Goldstein's demonstration of the dissociation of an enzyme-inhibitor complex on dilution was based upon equilibrium considerations. We are now in a position to investigate the rate at which this occurs. If it is infinitely slow, it is of no practical consequence to the experimenter. If it is moderately slow, and determinations are made upon diluted serum before dissociation is complete, neither the uncorrected nor the fully corrected (for dilution) data will be accurate. Dilution corrections can only be applied *in toto* if the dissociation is very rapid, or if the diluted serum is allowed to stand long enough before determination for equilibrium to be achieved.

Let us again write the equation for change of (EI) with time:

$$\frac{d(E')}{dt} = k_1(E_f)(I_f) - k_2(E')$$

Letting $E_f/E \equiv a$ (since S is absent),

$$I_f \doteq I \text{ (zone } A\text{)}$$
$$EI = (1 - a)E$$

then,

$$\frac{da}{dt} = -k_1[(I + K_I)a - K_I] \tag{19A}$$

Now let I before dilution be I_0, and let I after dilution be NI_0, where N is the factor of dilution. Integrating equation 19A, making the above substitutions, and setting $I = I_0$ when $t = 0$, we arrive at:

$$a = \frac{K_I}{NI_0 + K_I}\left\{1 + \left(\frac{NI_0 + K_I}{I_0 + K_I} - 1\right)e^{-k_1 t(NI_0 + K_I)}\right\} \tag{20}$$

We may eliminate I_0 entirely by substituting its original non-competitive value

$$I_0 = \frac{K_I(1 - a_0)}{a_0}$$

to get an equation relating a at time t to a_0 before dilution.

$$a = \frac{a_0}{N - (N - 1)a_0} \left\{1 + [N - (N - 1)a_0 - 1]e^{-\frac{k_2 t[N-(N-1)a_0]}{a_0}}\right\} \qquad (21)$$

From equation 21 one can plot theoretical dilution dissociation curves in zone A for any dilution factor N and any initial activity a_0. In Fig. 10 we have done this for two initial activities in serum, diluted to 4.54 per cent and 9.09 per cent, using the value of k_2 obtained previously. The two pairs of solid curves in the lower part of the graph show this and the points represent data from an experiment designed to confirm this slow effect of dilution. In the experiment, serum containing inhibitor was diluted to the desired concentration at zero time, placed at 38°C. within 20 minutes, incubated for 20 minutes, and then determined against substrate which was introduced at various times. The points given are the observed activities; they are undoubtedly too high because they are 3 to 23 minute readings and therefore subject to the competition error already discussed; and there should also be a small correction for destruction of physostigmine during the experiment. The fit is therefore not too satisfactory, but the general magnitude of the time course certainly agrees with that predicted by the curves.

The pair of curves in the upper part of the graph is purely theoretical and is illustrative of the course of events on dilution to 22.2 per cent and 1.0 per cent of serum with 56.3 per cent of normal activity initially (one-tenth the inhibitor concentration used in the upper of the lower pairs of curves).

The time course of dissociation on dilution is seen to be moderately slow. The dilution correction must be applied to experimental results with this fact in mind, and experiments should preferably be designed with an equilibration period *after* dilution long enough for dissociation to be completed.[8]

B. Kinetics of Combination.—

A number of investigators have observed that the attainment of equilibrium between physostigmine and cholinesterase is a slow process, and various expedients, such as storage overnight in the ice chest, have been adopted to insure

[8] The solution of the dilution-time problem for zone B follows the same steps as above but nothing would be gained by presenting the equation (which involves an integral of the form $\int \frac{da}{Ca^2 + Ba + A}$).

complete equilibration before any determinations are made. No quantitative work has come to our attention describing *how* slowly the combination proceeds, nor how great an error is involved in determining activity at some time before equilibrium.

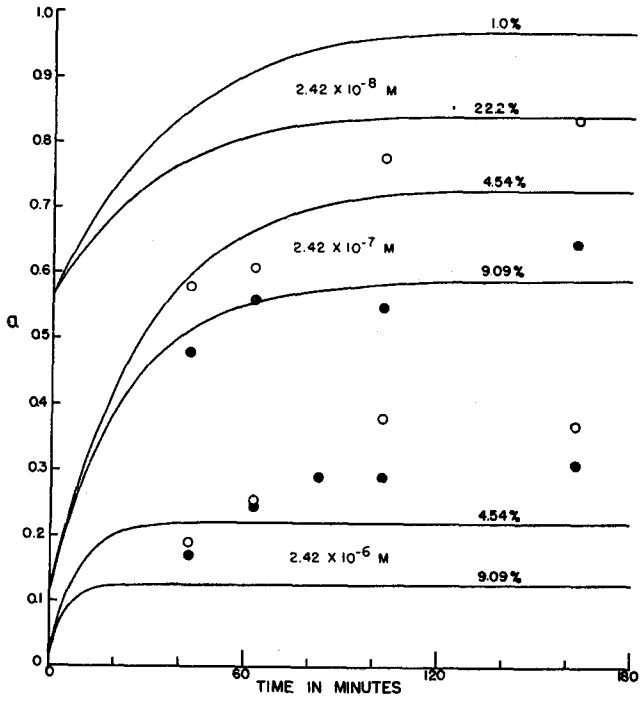

FIG. 10. Kinetics of the dilution effect. The two lower pairs of curves represent the theoretical time course of the dilution effect, for $k_2 = 0.026$, when sera containing 2.42×10^{-7} M and 2.42×10^{-6} M physostigmine are diluted at zero time to 4.54 per cent and 9.09 per cent. ● = observed activities (plotted at the midpoints of the 20 minutes periods) for 9.09 per cent. ○ = observed activities for 4.54 per cent. These points are not corrected for destruction or competition, so that all values of a are too high. The upper pair of curves shows the theoretical course of the reaction when serum containing 2.42×10^{-8} M physostigmine is diluted to 22.2 per cent and to 1.0 per cent.

Combination is obviously determined by the velocity constants k_1 and k_2. This combination rate is given by equation 19*A*, which may be integrated directly, setting $a = 1$ when $t = 0$, giving:

$$a = \frac{1}{I' + 1} \{1 + I' e^{-k_2 t (I'+1)}\} \tag{22}$$

Using the value of k_2 already obtained we have plotted, in Fig. 11, the theoretical curves showing the course of combination of added inhibitor with enzyme in zone A. This time course is in substantial agreement with our own experience and with the results which have appeared in the literature. The curves show, as would be expected, that combination is almost immediate with high concentrations of I and very slow with low concentrations. The error

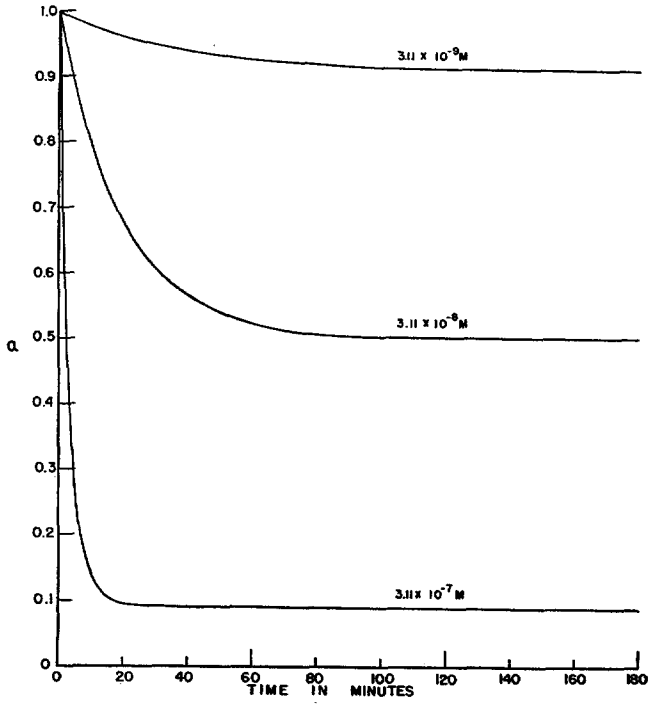

FIG. 11. Combination of inhibitor with enzyme. The theoretical time course of the reaction, for $k_2 = 0.026$, when three concentrations of physostigmine are added to dog serum (zone A).

in determining a at, let us say, one-half hour would be greatest (about 0.07) for medium values of I.

The time course of combination is independent of E in zone A. For zone B, it can be obtained by integrating, with respect to a, the equation:

$$\frac{da}{dt} = -k_2[E'a^2 + (I' - E' + 1)a - 1] \qquad (19B)$$

Destruction of Inhibitor or Substrate.—

We shall consider the enzymatic destruction of inhibitor, bearing in mind that we are thereby treating it as a substrate, and that consequently the same

considerations will apply to substrate breakdown itself. Inhibitor destruction may be described as follows:

$$E + I \rightleftharpoons EI \xrightarrow{k_D} E + \text{split products}$$

where k_D is the velocity constant of destruction. Then,

$$\frac{dI}{dt} = -k_D(EI) \tag{23}$$

Substituting,

$$I = K_I \frac{(1 - a)}{a} + (1 - a)E$$

$EI = (1 - a)E$, and the other substitutions as usual:

$$\frac{da}{dt} = \frac{k_D(1 - a)}{\frac{1}{E_I' a^2} + 1} \tag{24}$$

Integrating, and setting $a = a_0$ when $t = 0$:

$$\left[\frac{1}{a_0} + ln\ \frac{1 - a_0}{a_0} - E_I'\ ln(1 - a_0)\right] - \left[\frac{1}{a} + ln\ \frac{1 - a}{a} - E_I'\ ln(1 - a)\right] = k_D E_I' t \tag{25}$$

In this equation the terms on the left containing E_I' are insignificant even when E_I' is considerably greater than 0.1, so it is safe to reduce it to the zone A form:

$$\left[\frac{1}{a_0} + ln\frac{1 - a_0}{a_0}\right] - \left[\frac{1}{a} + ln\ \frac{1 - a}{a}\right] = k_D E_I' t \tag{25A}$$

Equation 25A allows one to plot the theoretical curve of destruction starting with any initial activity a_0, as a function of $k_D E_I' t$. We then require an experimental curve to which we can adjust the time scale, a procedure which at the same time gives the best fit of the data to the theoretical curve and also the best value for the term $k_D E_I'$. There is no way of solving for either of these constants separately by means of the destruction function alone. If E_I' can be determined by other means, we will then know k_D, but in the series of experiments described in this paper we have shown that it is impossible to settle on any but a maximum value of E_I' (< 0.58 for cholinesterase in 4.54 per cent dog serum).

Ellis, Plachte, and Straus (17) made a study of the course of physostigmine destruction in horse serum and plotted the increase of a with time in their Fig. 4. Accepting their experimental data and fitting our theoretical curve (be-

ginning with $a_0 = 0.08$) to their "corrected serum curve," we obtain curve B of Fig. 12, which is an almost identical fit to their data. When such a fit is made, the value obtained for $k_D E_I'$ is 1.4. Using this value we have plotted

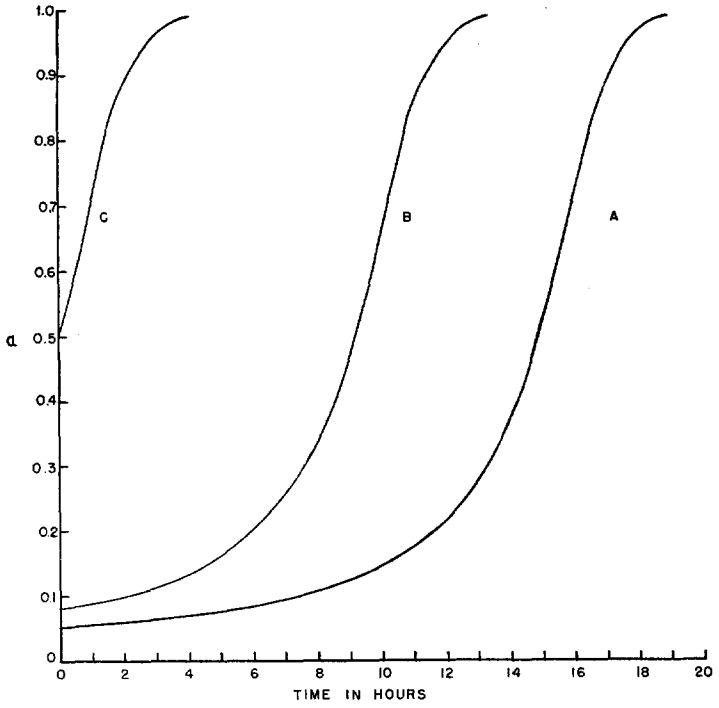

FIG. 12. Enzymatic destruction of inhibitor or substrate. The theoretical time course of the increase in fractional activity is shown for different initial activities. The time scale is so chosen that the experimental data of Ellis, Straus, and Plachte for cholinesterase and physostigmine will be fitted by curve B. The resulting destruction constant, $k_D = 0.00182$ minutes^{-1}, is used to plot curves A and C. All possible destruction curves with initial activity greater than 0.05 are really segments of curve A, which is the major part of the single curve that represents the course of this reaction. These curves could be generalized by using $k_D E't$ as abscissa, and made valid for substrate by then substituting $(1 - a)$ for a on the ordinal axis (see text).

similar curves (A, C of Fig. 12) beginning at different initial activities. These, however, are actually all segments of a *single* destruction curve, represented in its major part by curve A. That all the other curves should be identical with some segment of curve A arises from the fact that the destruction rate is proportional to the activity a at any time, regardless of the previous history of the reaction.

This destruction curve, which applies to the combination of a single molecule

of inhibitor with a molecule of enzyme, is characterized by an infinitely slow increase in a when a is practically zero (although *absolute rate of inhibitor destruction* is most rapid here). The rate of change of a slowly increases, becoming quite rapid at about 0.20. The most rapid change occurs at 0.667,[9] where the curve inflects; and very close to maximal activity the slope falls off quite rapidly, the rate of change of a becoming again infinitely slow as a approaches 1.

For the change in a when substrate is being destroyed, the reasoning is identical, giving the same curve, except that $(1 - a)$ must be substituted for a in all the equations and on the ordinate of the destruction-time curve, the time scale being determined by k_D for the given substrate.

Ellis, Plachte, and Straus studied the spontaneous as well as the enzymatic breakdown of physostigmine, and deriving the equation

$$k_D = \frac{2.3}{at} \log \frac{b}{b - x} \tag{26}$$

plotted $\log \frac{b}{b - x}$ against time to obtain an estimate of $k_D a$. Here b represents initial physostigmine, x the amount destroyed, and a the concentration of the other reactant—either enzyme or hydroxyl ion. They found that while the expected linear plot was obtained for the hydrolytic breakdown, the enzymatic reaction yielded a linear curve only after 6 hours. Their conclusion was that both this phenomenon and the observed slow rate of change of a at the beginning of the destruction-time curve represented an "inhibition due to excess substrate (physostigmine)."

But we have already demonstrated that the initial slow change in a is perfectly typical of the expected reaction mechanism and that there is no need to assume inhibition by excess substrate. The incorrect conclusion arrived at by these authors can be shown to arise from an invalid approximation in the derivation of their equation for enzymatic destruction of inhibitor.

Equation 26 is derived from the relation:

$$dx/dt = k_D\, ba \tag{27}$$

In our terminology this amounts to:

$$-dI/dt = k_D(OH^-)I, \quad \text{for hydrolysis} \tag{28}$$

and

$$-dI/dt = k_D \cdot E \cdot I, \quad \text{for enzymatic breakdown.} \tag{29}$$

Let us first take up equation 29, in which the rate is said to depend upon the product of inhibitor by *total* enzyme concentration—the common "monomolecular assumption." We know that the rate in fact depends upon *com-*

[9] This is arrived at by setting $d^2a/dt^2 = 0$ in equation 24.

bined enzyme concentration, according to equation 23. These two equations are only truly *equivalent* when $E \cdot I = (EI)$ and this is never the case for any real values of a. However, they *are* of the same *form* if a linear function relates (EI) to E and I, so that

$$(EI) = c \cdot E \cdot I \quad \text{for then equation 29 becomes}$$

$$-dI/dt = \frac{k_D}{c}(EI) = k_D'(EI)$$

Thus it is legitimate to assume a monomolecular reaction mechanism for the destruction of inhibitor (or substrate) by enzyme only if (1) there is no intermediate and the reaction is irreversible; or (2) a reversibly formed intermediate breaks down so rapidly that its existence may be neglected; or (3) an intermediate, whose concentration may be appreciable, is formed by the stoichiometric combination of the initial reactants. The monomolecular reaction mechanism does not apply under other circumstances which are frequently encountered. Specifically, in the case under discussion, the concentration of the intermediate (EI) is appreciable and its reversible formation from E and I is not stoichiometric; consequently, the destruction rate cannot be assumed to depend upon the concentrations of the initial reactants, but only upon that of (EI).[10]

The effect of this fallacy in the theoretical premises of the above cited authors may be illustrated by repeating for the correct equation 23 the steps of substitution and integration which were used to derive their equation 26 from the incorrect equation 29, yielding:

$$k_D = \frac{2.3K_I}{at}\left[\log \frac{b}{b - x} + \frac{x}{2.3K_I}\right] \tag{30}$$

If $\log \frac{b}{b - x}$ is now plotted against time we shall obviously have a curve which is not linear but deviates progressively from linearity—as a matter of fact this equation now perfectly describes the experimental points of Ellis, Plachte, and Straus' Fig. 4. In other words both their predication of a linear plot and their assumption of an unusual reaction mechanism (inhibition by excess inhibitor) to explain the observed non-linearity are unwarranted, the reaction actually following the expected theoretical course without deviation.

As we should expect from the foregoing discussion, the value of k_D obtained by these authors (2.74×10^6) has no relation to the correct constant, for not only its magnitude but its dimensions are different. For our maximum value

[10] The reader is referred here to Northrop's (18) discussion of this question and his conclusion that in the trypsin-gelatin-trypsin inhibitor system an enzyme-inhibitor but not an enzyme-substrate complex is formed.

of E_I' (12.8 in whole serum), $k_D = 0.00182$. Since E_I' may be much smaller, as we have shown, this must be regarded as the minimum value of k_D.

It is interesting in view of the discrepancies arising from the use of equation 29 that equation 28, which resembles it superficially, should give such satisfactory results (*cf.* Figs. 3*A* and 3*B* of the above authors). The reason for this is that the two equations are not really analogous, for the term (OH^-) is not *total* but *free* hydroxyl.[11] Because this equation is stated only in terms of a *free* reactant, no false assumptions are involved and it is perfectly valid. Equation 29 would be equally correct if written in terms of *free* rather than *total* enzyme;[12]

$$-\frac{dI}{dt} = k_D \cdot E_f \cdot I = k_D(aE)\left(\frac{1-a}{a}\right) = k_D(1-a)E = k_D(EI)$$

and it has become identical with the legitimate zone *A* equation 23.

SUMMARY

The mechanism of enzyme-inhibitor-substrate reactions has been analyzed from a theoretical standpoint and illustrated by data from the system cholinesterase-physostigmine-acetylcholine. This treatment is by no means limited to a single system but should be generally applicable to others of similar type.

Competitive enzyme-inhibitor-substrate systems show the same characteristic "zones of behavior" already demonstrated for non-competitive systems by Straus and Goldstein. These zones, three in number, determine the mathematical function which relates activity of an enzyme to concentration of an added substrate or inhibitor or both.

The effects of suboptimal substrate concentration in such systems have been considered, and the errors arising from various common simplifications of the descriptive equations have been pointed out.

The zone behavior phenomenon has been shown to be useful in determining the number of molecules of substrate or inhibitor combining reversibly with a single enzyme center.

The kinetics of competitive inhibition, dilution effect, combination of inhibitor or substrate with enzyme, and destruction of inhibitor or substrate by enzyme have been analyzed and experimentally verified, and absolute velocity constants have been determined.

Theoretical conclusions have been discussed from the standpoint of their physiological significance.

[11] We determine the *free* (OH^-) but do not measure the *total* hydroxyl, including that which has combined with *I*.

[12] This recalls the footnote discussion in Straus and Goldstein, page 568, on the Henderson-Hasselbalch equation.

Specifically, it has been shown that:

1. The inhibition of cholinesterase by physostigmine is competitive. A single molecule of physostigmine or acetylcholine combines with one center of cholinesterase—$n = 1$; and the mechanism $n = 2$ has been excluded. Numerical values of the constants for this system are as follows:[13]

$$K_I = 3.11 \times 10^{-8}$$
$$k_1 \text{ (combination)} = 8.3 \times 10^5$$
$$k_2 \text{ (dissociation)} = 0.026$$
$$K_S = 1.25 \times 10^{-3}$$
$$k_3 \text{ (combination)} = 260$$
$$k_4 \text{ (dissociation)} = 0.32$$

2. No definitive value can be assigned to E, the molar concentration of enzyme centers, but in 4.54 per cent dog serum, $E < 1.8 \times 10^{-8}$ ($E_I' < 0.58$). The system therefore operates in (or nearly in) zone A at this concentration.

3. Competitive displacement of inhibitor by substrate and *vice versa* introduces considerable error in the usual 20 minute determination of the activity of an inhibited enzyme, unless properly corrected for.

4. Dissociation of the enzyme-inhibitor complex on dilution proceeds moderately slowly so that the full corrections for dilution cannot be applied unless time has been allowed for full dissociation.

5. Combination of physostigmine with cholinesterase is slow at all but large concentrations of inhibitor.

6. The destruction of physostigmine or acetylcholine by cholinesterase follows the predicted curve; k_D for the destruction of physostigmine is found to be > 0.00182; k_D for acetylcholine destruction is > 3500.[13] There is no reason to assume inhibition of destruction by excess substrate or inhibitor.

7. The common assumption that enzymatic activity follows (or nearly follows) a monomolecular course is true only under limited conditions, which have been here defined. It is not valid, as a rule, for the enzymatic destruction of an inhibitor (*e.g.*, physostigmine) and its application to such a case may lead to erroneous conclusions about the reaction mechanism.

The author is deeply indebted to Dr. Otto Krayer for the interest, support, and inspiration which catalyzed this work through its every phase. Sincere thanks are also due Dr. John T. Edsall for his unstinting advice, criticism, and encouragement; Dr. Oliver H. Straus, with whom much of the basis for the present material was first conceived; Mr. Eliot Silverman for assistance with the mathematical sections; and Ruth Silber for invaluable aid in preparation of the manuscript.

[13] All constants are expressed in terms of mols, liters, and minutes.

APPENDIX

TABLE II

Acetylcholine Activity Curve

(Data for Fig. 1)

Concentration of acetylcholine bromide (mols per liter reaction mixture)	Average observed velocity (c.mm. CO_2 per 20 min.)	
2.02×10^{-1}	65.7	(1)*
1.61×10^{-1}	64.5	(5)
8.06×10^{-2}	63.1	(2)
5.04×10^{-2}	61.0	(1)
4.04×10^{-2}	61.3	(3)
2.02×10^{-2}	58.1	(2)
1.26×10^{-2}	51.5	(1)
1.01×10^{-2}	55.0	(3)
5.04×10^{-3}	49.8	(2)
3.16×10^{-3}	43.8	(1)
2.54×10^{-3}	47.3	(1)
1.26×10^{-3}	33.7	(2)
7.90×10^{-4}	25.5	(1)
6.34×10^{-4}	24.9	(1)
3.16×10^{-4}	11.0	(2)
1.97×10^{-4}	7.1	(2)
1.58×10^{-4}	8.3	(2)
Non-enzymatic hydrolysis		
1.0×10^{-2}	0.25	(1)
1.26×10^{-2}	0.75	(1)
4.0×10^{-2}	3.50	(1)
5.0×10^{-2}	3.50	(1)
1.62×10^{-1}	20.0	(1)
2.0×10^{-1}	21.5	(1)

* Numbers in parentheses indicate the number of determinations included in the average.

TABLE III

(Data for Fig. 3)

Curve C	
Concentration of physostigmine salicylate (mols per liter reaction mixture)	a
8.7×10^{-8}	0.95*
3.8×10^{-7}	0.85
7.8×10^{-7}	0.75*
8.3×10^{-7}	0.73*
1.7×10^{-6}	0.52*
3.1×10^{-6}	0.39
3.3×10^{-6}	0.36*
4.4×10^{-6}	0.33
6.6×10^{-6}	0.22*
8.7×10^{-6}	0.18
1.3×10^{-5}	0.10*
2.4×10^{-5}	0.08

Curve OBS	
Concentration of physostigmine salicylate (mols per liter reaction mixture)	a (based on velocities 3 to 23 min. after S added)
1.1×10^{-9}	~1.00
2.2×10^{-9}	0.94*
4.4×10^{-9}	0.90
5.5×10^{-9}	0.83*
6.05×10^{-9}	0.90
1.1×10^{-8}	0.83
1.1×10^{-8}	0.80
2.2×10^{-8}	0.66*
2.76×10^{-8}	0.63
5.5×10^{-8}	0.41*
5.5×10^{-8}	0.44
5.5×10^{-8}	0.46
6.05×10^{-8}	0.48
1.1×10^{-7}	0.33
1.1×10^{-7}	0.33
2.2×10^{-7}	0.25*
5.5×10^{-7}	0.23
5.5×10^{-7}	0.23*
1.35×10^{-6}	0.17*
5.5×10^{-6}	0.18*
5.5×10^{-6}	0.12
6.05×10^{-6}	0.14

* Indicates average of satisfactory duplicate determinations.

TABLE IV

Displacement of S by I

(Data for Fig. 8)

(Min. after *I* added)	*I*(mols per liter reaction mixture)	
	1.1×10^{-6}	1.1×10^{-5}
t	*a*	*a*
5.5	0.93	0.58
10.5	0.90	0.30
15.5	0.86	0.28
20.5	0.78	0.14
28	0.59	0.09
38	0.67	0.13
48	0.63	0.09
58	0.74	0.11
68	0.61	0.11
78	0.55	0.09
88	0.57	0.10
98	0.56	0.11
108	0.66	0.10
118	0.60	0.13

TABLE V

Displacement of I by S

(Data for Fig. 9)

(Min. after *S* added)	*I*(mols per liter reaction mixture)				
	5.5×10^{-6}	1.35×10^{-6}	5.5×10^{-7}	5.5×10^{-8}	5.5×10^{-9}
t	*a*	*a*	*a*	*a*	*a*
13	0.14	0.17	0.23	0.41	0.83
33	0.20	0.30	0.49	0.60	0.90
53	0.25	0.46	0.60	0.73	0.96
73	0.21	0.43	0.66	0.80	0.98
93	0.21	0.45	0.74	0.81	0.99
113	0.24	0.48	0.78	0.92	1.02
133	0.22	—	0.74	0.93	—
153	0.23	—	0.82	0.92	—
173	0.25	—	0.81	0.93	—
223	—	0.50	—	—	—
243	—	0.54	—	—	—

TABLE VI

Time Course of Dilution

(Data for Fig. 10)

Observed activities (3 to 23 minutes after S addition), uncorrected for competition or destruction.

(Min. after dilution)	$I = 2.42 \times 10^{-7}$ mols per liter serum	
t	*9.09 per cent*	*4.54 per cent*
43	0.48	0.58
63	0.56	0.61
103	0.55	0.78
163	0.65	0.84
283	0.68	0.97
	$I = 2.42 \times 10^{-6}$ mols per liter serum	
43	0.17	0.19
63	0.25	0.25
83	—	0.29
103	0.29	0.38
163	0.31	0.37
253	—	0.42
283	0.24	—

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