# ZONE BEHAVIOR OF ENZYMES

## Illustrated by the Effect of Dissociation Constant and Dilution on the System Cholinesterase-Physostigmine*

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### INTRODUCTION

The study of systems composed of an enzyme and reversible inhibitor has contributed greatly to our knowledge of enzyme kinetics. Such systems are of importance in the field of pharmacology, since a number of drugs act by inhibiting known enzyme systems, while many of those whose biochemical mode of action is still unknown may operate in similar fashion.

The classical treatment of the kinetics of enzyme reactions has been based upon the assumption of a very small concentration of enzyme centers acting according to the laws governing first order reactions (pseudomonomolecular). In this paper we shall show that under a number of common conditions such treatment cannot adequately describe the behavior of the system but that a more complete analysis must be employed. Enzyme-inhibitor and enzyme-substrate systems will be shown to behave in three distinct ways depending upon the concentrations of the reactants and the dissociation constant of the system. The boundaries of these three "zones of behavior" will be established on a kinetic basis applicable to all such systems, and the qualitative and quantitative differences in behavior will be demonstrated.

An important practical consequence of the theory of zone behavior concerns the effect of diluting a mixture of enzyme and inhibitor (or substrate). It is common practice to remove serum from an animal which has received some drug, and then, after appropriate dilution, to determine *in vitro* the degree of inhibition produced in some serum enzyme. It is then assumed that the observed degree of inhibition obtained in this manner is representative of the state of the enzyme in the animal's circulating serum before removal. It will be shown, however, that dilution is a crucial operation which significantly affects the subsequent experimental observations, and that a conversion equation (or conversion curves) must be used if the usual experimental data are to be applied to the situation existing *in vivo*.

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The general methods presented in the first part of this paper will then be applied in an illustrative fashion to the system cholinesterase-physostigmine, which will be shown both qualitatively and quantitatively to exhibit the predicted behavior on dilution.

In the final section we shall discuss: (*a*) the evolution of the concept of zone behavior; (*b*) further conclusions of biological importance; (*c*) an alternative definition of the zone boundaries; (*d*) extension of the analysis to more complex systems; and (*e*) certain significant limitations on the analysis here presented.

## THEORETICAL

### *Derivation of the General Equation*

In studying any enzyme from a kinetic standpoint, the observed data are necessarily based upon (*a*) the concentration of a substance, $X$, that combines with the enzyme, $E$, to form a complex, $EX$; and (*b*) the rate of reaction at which breakdown products, $Sp$, are formed from $EX$ with the liberation of $E$. The substance, $X$, may be either a substrate or an inhibitor, depending on the behavior of the complex, $EX$; and it is necessary to define precisely what is meant by these terms.

Let us consider the combination of enzyme $E$ with a single molecule of $X$ to form a complex $EX$:

$$E + X \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EX \xrightarrow{k_3} E + Sp, \qquad \text{(reaction A)}$$

where $k_1$, $k_2$, and $k_3$ are velocity constants, and $Sp$ represents the split products of $EX$ breakdown. In this reaction we call $X$ a substrate if the complex $EX$ breaks down to form $Sp$ at a rate that is *not* negligible for the purposes under consideration. If, on the other hand, the breakdown of $EX$ *is* negligible ($k_3 \ll k_2$), we call $X$ a reversible inhibitor.

It is evident from this that the difference between a substrate and a reversible inhibitor is determined only by the relation of the velocity constants in reaction A. It also follows that all reversible inhibitors whose action is upon the same enzyme centers as normally would combine with substrate molecules are necessarily *competitive* inhibitors. The *degree* of competition will naturally vary, but whether it be considerable (as when one substrate "inhibits" the breakdown of another) or very slight (as when a potent drug combines with a substantial number of enzyme centers), there is no basic difference in the kinetic mode of action. For purposes of simplicity, however, this paper will limit itself to the case where competition is negligible or truly absent (see Discussion, p. 583).

Let $E$, $I$, $S$, $EI$, and $ES$ now represent the total molar concentrations of enzyme centers, inhibitor, substrate, and their complexes respectively. Then,

if $v$ is the observed velocity of substrate breakdown, it is true under all circumstances that

$$v = k_3(ES)$$

The concentration of complex, $ES$, cannot be measured directly. If, however, a large excess of substrate is added, in accordance with the principle of mass action, virtually all the enzyme will combine with it to form the complex, $ES$, so that $ES \doteq E$; and the enzyme remaining free, $E - ES \doteq 0$. Under these circumstances, further increase in $S$ can produce no increase in $ES$, and so no increase in $v$; then

$$v_{max.} = k_3 E$$

If an inhibitor be present, a fraction of the total enzyme will combine with it to form the inactive complex, $EI$, and the amount of enzyme left free to combine with an excess of substrate will be $(E - EI) = ES$. Substituting this value of $ES$,

$$v = k_3(E - EI)$$

and then dividing one equation by the other,

$$\frac{v}{v_{max.}} = \frac{k_3(E - EI)}{k_3 E} = 1 - \frac{EI}{E}$$

If we now let $i$ represent the fraction of the total enzyme that is combined with inhibitor,

$$i \equiv \frac{EI}{E}$$

then from the above,

$$i = 1 - \frac{v}{v_{max.}} \qquad (1)$$

The fractional inhibition, $i$, of an enzyme can therefore readily be found, since both $v$ and $v_{max.}$ are measurable quantities; $i$ will vary between the limits 0 and 1 as $v$ varies from $v_{max.}$ to 0.

The reaction between enzyme and inhibitor (reaction A) becomes entirely equivalent to

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \qquad \text{(reaction B)}$$

since breakdown of the combined form is negligible. If the law of mass action is followed, then at equilibrium,

$$\frac{(E - EI)(I - EI)}{(EI)} = \frac{k_2}{k_1} = K$$

where $K$ is the dissociation constant of the complex. Substituting the value $EI = iE$ (since $i \equiv EI/E$), and simplifying,

$$I = \frac{Ki}{1 - i} + iE \tag{2B}$$

This equation states that the *total* molar concentration of inhibitor ($I$) is equal to the sum of two parts. One of these, $iE$, will be recognized as equivalent to $EI$, the molar concentration of *combined* inhibitor. It follows that the other part, $\frac{Ki}{1 - i}$, must represent the molar concentration of *free* inhibitor. Equation $2B$ then says simply that total inhibitor equals free plus combined forms.

Now it will be obvious that if the enzyme concentration[1] is very small practically all the inhibitor is present in the free form. On the other hand, if enzyme concentration is very great, nearly all the inhibitor will be in the combined form (except at extreme values of $i$). It should thus be possible to introduce working simplifications of the equations by neglecting combined inhibitor, on the one hand,

$$I = \frac{Ki}{1 - i} \tag{2A}$$

or free inhibitor, on the other,

$$I = iE \tag{2C}$$

for each of the two cases considered. However, it is clear from inspection of the equation that these simplifications cannot really be justified on the basis of the actual magnitude of the enzyme concentration $E$, but rather by its magnitude *relative to* $K$.

The dissociation constant $K$ here has the dimensions of concentration and is usually expressed in molar units. It is a constant for any given enzyme-substrate or enzyme-inhibitor system, provided only that all the physical conditions not mentioned in the equation, such as temperature, pH, choice of reactants, and so on, are held constant. Conversely, $K$ may vary continuously if temperature or pH changes; or discontinuously if one enzyme, substrate, or inhibitor is substituted for another.

The use of simplified forms of the equation describing the kinetic behavior of *all* enzyme-inhibitor systems of the general type under consideration has just been shown to depend upon the ratio $E/K$, and not upon absolute concentrations of enzyme or inhibitor. If we therefore express $E$ and $I$, not in molar concentrations, but *using $K$ as our unit* for whatever system we deal

[1] $E$ is the total molar concentration of enzyme *centers*, irrespective of the number of centers that may be carried by a single protein molecule.

with, we will thereby generalize our discussion to apply to any enzyme-inhibitor system.[2]

The term $E/K$ we shall call the "specific concentration" of enzyme and designate as $E'$[3]. Similarly, $I/K$ is the "specific concentration" of inhibitor, designated by $I'$.

Dividing equation 2 $B$ by $K$ and substituting, we now have

$$I' = \frac{i}{1-i} + iE' \tag{3B}$$

For the case where specific concentration of enzyme ($E'$) is small, and practically all the inhibitor is free, this becomes

$$I' = \frac{i}{1-i} \tag{3A}$$

It is evident that in this case the inhibition is a function of specific concentration of inhibitor alone, and independent of enzyme concentration.

For the case where $E'$ is large and practically all the inhibitor is combined, the equation becomes

$$I' = iE' \tag{3C}$$

Here the inhibition is a function of specific concentration of inhibitor and enzyme, being equal to the ratio $I'/E'$.

We now see that equation 3 in its three forms describes the behavior of all enzyme-inhibitor systems acting according to reaction B. Furthermore, since nothing has been added which is not implicit in the mass action law, it is equally valid for any system—chemical, physical, or biological—where two reactants combine reversibly in a manner described by this law.

### *Zones of Enzyme Behavior*

The three forms of equation 3 represent three distinct zones of enzyme behavior, hereafter designated A, B, and C, after the equations which define them. It will be necessary now to determine more exactly the boundaries of these three zones of behavior. How "large" or how "small" must $E'$ be in order that equation 3$A$ or 3$C$ instead of the full form 3$B$ may be used to describe behavior adequately? The answer will depend upon how large an error in $i$ (our experimentally measured term) we are willing to accept. Having decided upon the maximum acceptable error ($\Delta i$), we can then determine where the zone boundaries must lie in order that this error shall never be exceeded. It should

[2] It will be recalled that $i \equiv EI/E$ is a dimensionless number and hence is unaffected by changing the system of units employed.

[3] $E'$ and $I'$, being ratios of concentration, are dimensionless numbers. The term "specific concentration" is arrived at by analogy to specific gravity (the measure of density relative to that of water, taken arbitrarily as unity).

be evident that the more rigid we are in fixing $\Delta i$, the larger will be the zone in which the full equation $3B$ must be used.

Fig. 1 shows the zone boundaries for three arbitrary values of $\Delta i$. Strictly interpreted, zone B lies between the pairs of boundary curves for any given $\Delta i$, and the zone boundaries are seen to vary with the fractional inhibition $i$. For working purposes, it is necessary to eliminate this variation with $i$ and decide

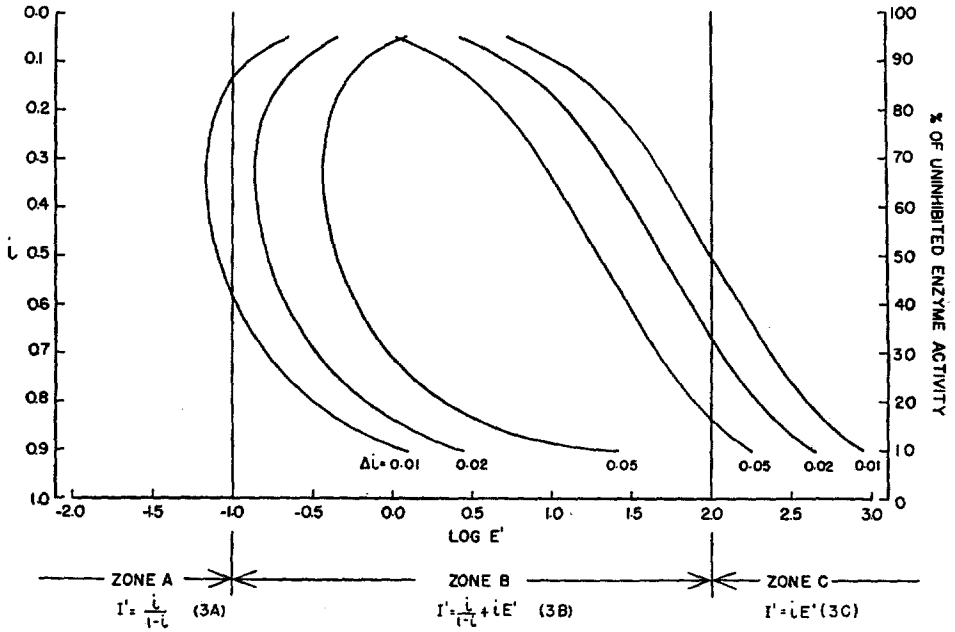


FIG. 1. Zone boundaries. Ordinate, fractional inhibition, $i$. Abscissa, $\log_{10}$ of the specific enzyme concentration ($E'$). Each pair of curves shows the exact boundary of zone B for a given value of $\Delta i$. To the left of the region enclosed by each pair lies zone A; to the right, zone C. Straight vertical lines are approximate zone boundaries, when $\Delta i = 0.01$, neglecting the effect of variation of $i$ on the boundary value of $E'$.

upon two boundary values of $E'$ which will give the best approximation. In the case illustrated, $\Delta i = 0.01$ was selected, and approximate boundary values are shown to be $E' = 0.1$ to the left and $E' = 100$ to the right.

The mathematical derivation of the boundary curves plotted on Fig. 1 is as follows: At the boundary $AB$, $I'$ in equation $3A$ plus the error caused by the increment $\Delta i$ must equal $I'$ in equation $3B$.
Thus,

$$\frac{i + \Delta i}{1 - (i + \Delta i)} = \frac{i}{1 - i} + iE'$$

or

$$E' = \frac{\Delta i}{i} \cdot \frac{1}{(1 - \Delta i - i)(1 - i)} \qquad \text{at boundary } AB.$$

At boundary $BC$, $I'$ in equation $3C$ plus the error caused by the increment $\Delta i$ must equal $I'$ in equation $3B$, so that

$$(i + \Delta i)E' = \frac{i}{1 - i} + iE'$$

and

$$E' = \frac{i}{\Delta i} \cdot \frac{1}{1 - i} \qquad \text{at boundary } BC.$$

*Most Enzyme Systems Operate in Zone A.*—Most of the general treatments of enzyme kinetics have hitherto been based on the assumption that the concentration of enzyme centers is constant and so small compared with the concentration of any substance with which it may combine that it may be neglected. This is the situation to which equation $3A$ has been shown to apply. Michaelis and Menten (1), Haldane (2), Lineweaver and Burk (3), and others have all based their algebraic and graphic treatment upon this assumption and consequently have limited their discussions to zone A. Likewise the familiar Michaelis law applies only within this zone.[4]

Their failure to extend their fertile methods to zones B and C is due to the fact that most enzymes are studied in very dilute solution. There are several reasons for this. First, enzymes are considered to be protein molecules carrying only one or very few active centers per molecule, so that the factor of solubility precludes high molar concentrations of enzyme centers. Second, even if the enzyme can be concentrated to some degree, it is seldom technically convenient to measure the very high reaction velocities that occur in concentrated solution under optimal conditions. A survey of any list of dissociation constants such as that given by Haldane (2),[5] will show that in the great majority of instances $K$ is greater than $10^{-5}$ molar; with $E$, the concentration of enzyme, limited by the considerations just mentioned, $E/K$ or $E'$ will be less than 0.1, so that the systems lie in zone A.

*Systems in Zones B and C.*—

1. When $K$ is small: Since $E'$, which determines the zone of an enzyme system, is defined as $E/K$, it is clear that if $K$ is small enough $E'$ may be between 0.1 and 100, so that the system is in zone B, or may be greater than 100,

[4] The Michaelis law states that the concentration of inhibitor required for half inhibition is equal to the dissociation constant $K$; that is to say, $I = K$, or $I/K = 1$, and $I' = 1$, when $i = 0.5$. This is true only in equation $3A$.

[5] Haldane, J. B. S., Enzymes, London, Longmans, Green & Co., 1930, 35.

so that it is in zone C. A few enzyme-substrate complexes, such as peroxidases and "oxygenases," have dissociation constants of the order of $10^{-6}$ to $10^{-7}$, so that $E'$ might be greater than 0.1. Furthermore, a significant number of enzyme-inhibitor systems, exemplified by cholinesterase and physostigmine, have dissociation constants as small as or smaller than this. These may be expected to show zone B or C behavior *in vitro*. For a single enzyme studied at a single concentration, $K$ will in general be different for the various substances that form complexes with it, and $E'$ will vary inversely as $K$. Then for any two substances whose dissociation constants differ with respect to a single enzyme, it is possible that $E'$ may in one case be less than 0.1, and in the other case greater than 0.1. The system will then be in zone A with respect to the first substance and in zone B or C with respect to the second.

2. When $E$ is large: There is at least one situation where an enzyme may exist in relatively high concentration and yet not yield a reaction velocity that is technically unmanageable. Let us consider tissue slices or intact cells such as can be handled in the Warburg apparatus. These will be supposed to give conveniently measurable reaction velocities and to have been so handled that the enzyme distribution in the tissue has not been disturbed. There has accumulated much evidence that some enzymes, such as cholinesterase, are confined to a small fraction of the total number of cells, or are even confined to localized regions of a single cell. At these points of localization the molar concentration of enzyme centers may be very much higher than that indicated by a consideration of the total tissue or fluid volume involved. So long as the rate of the reaction measured is not limited by diffusion, the kinetic behavior might indicate that the system lay in zone B or C, even though the same total amount of enzyme would lie in zone A if it were dissolved throughout the total volume of the reaction mixture. If the differences of behavior exhibited by enzymes in the three zones could be experimentally detected, a means would be provided for estimating directly the enzyme concentration in the intact cell. Such differences of behavior will be pointed out below, together with certain practical tests for estimating specific enzyme concentrations.

### *Graphical Representation of the General Equation (3 B)*

*Description of Plot.*—The usual representation of the action of a drug upon its receptor *in vivo* or *in vitro* is the plot of effect as ordinate against the logarithm of the concentration of the drug as abscissa. Equation 3 $B$ is plotted in this way in Fig. 2, which shows the relation between the fractional inhibition $i$ and the logarithm of the specific concentration of inhibitor $I'$. Each curve represents this function at a single value of the specific concentration of enzyme $E'$, these values being chosen arbitrarily for convenient spacing of curves. It will be observed that the curves representing successively lower specific concentrations of enzyme are asymptotic to a limiting curve that is

nearly reached when $E'$ falls to 0.1. In the direction of increasing enzyme concentration the curves become steeper and parallel, the points of inflection occurring progressively nearer to the region where $i = 1.0$.

This figure provides a graphic example of the variations in behavior in each of the three zones, as previously discussed in connection with equation 3.

Zone A is represented by the limiting curve $E' \leqq 0.1$, this curve representing all values of $E'$ more dilute than 0.1. It follows from this curve that fractional inhibition depends only upon $I'$ and is independent of $E'$ within this zone. This plot also shows that $I = K$ ($I/K = 1 = I'$, log $I' = 0$) when $i = 0.5$, as postulated by Michaelis, only in this zone.

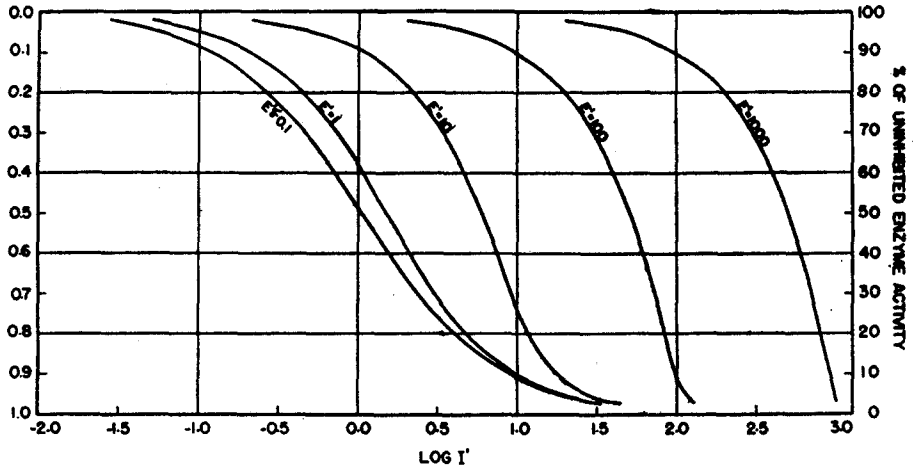


FIG. 2. Fractional inhibition, $i$, as a function of the $\log_{10}$ of the specific concentration of inhibitor $I'$ at various specific enzyme concentrations, $E'$.

Zone C is represented by the region to the right of the curve $E' = 100$. In this zone, the curves not only become parallel, but assume the shape of a simple logarithmic function. Furthermore, any two curves are separated by a distance which, measured off on the $I'$ axis, is equal to the factor by which $E'$ is changed between the two curves (*e.g.*, the horizontal distance from the curve $E' = 100$ to $E' = 1000$ is just 1 log unit on the $I'$ axis). As a consequence, the fractional inhibition ($i$) for a given $E'$ is directly proportional to the $I'$ employed, and the inhibition ($i$) is equal to the ratio of inhibitor to enzyme ($I'/E'$ or $I/E$) in the solution.

Zone B is, of course, represented by the area between the curves $E' \leqq 0.1$ and $E' = 100$, and here inhibition is a function of both $I'$ and $E'$, as stated by equation 3*B*.

*Slope: di/d log I'.*—The slopes of the curves of Fig. 2 are of interest because they provide a useful criterion for determining whether a system follows mass

law requirements, and also for roughly estimating the specific enzyme concentration.

If we consider the slope at $i = 0.5$, it will be evident from the form of the curves that this will be a minimum when $E' \leqq 0.1$, and a maximum when $E' \geqq 100$ (since the curves have attained their maximum steepness at this latter point). These limiting slopes are actually found to be 0.575 and 1.151.[6] Since these curves apply very generally to all systems of the type represented by reaction B, it follows that any such system in whatever zone must yield slopes within these limiting slopes at $i = 0.5$. Conversely, failure to fall within these limits is a result of only two possibilities: systematic or random experimental error is present, or the reaction does not follow this type of equilibrium equation.

If the slope falls within these limits, assuming that the reaction does follow this type of equilibrium equation, substitution of the experimentally determined slope in equation 4 (setting $i = 0.5$) will yield a preliminary value for $E'$ and hence an indication of the zone in which the system lies. Because a small change in slope corresponds to a large change in $E'$, the slope is of more use as an exclusion test than for precise evaluation of $E'$.

The reader may have noticed the agreement between the limiting slope 0.575 in zone A and Van Slyke's (4) maximum molar buffering capacity, $\beta_M$, of any monovalent buffer. This is not a coincidence but rather a reflection of the fact that, like equations 2 and 3, the Henderson-Hasselbalch equation is derived directly from the mass action law.[7]

### *Effect of Dilution*

We will now consider the effect of diluting an enzyme solution (*e.g.*, serum) containing a reversible inhibitor. It has long been realized that dilution of a

[6] The numerical values for slope are obtained by differentiating equation 3*B* with respect to log $I'$ and evaluating the limits when $E' \doteq 0$ and $E' \doteq \infty$. Thus:

$$\frac{di}{d \log I'} = 2.303i\left[1 - \frac{i}{1 + (1 - i)^2E'}\right] \qquad (4)$$

[7] Written in arithmetic form, the Henderson-Hasselbalch equation becomes $H^+ = \frac{K(1 - \alpha)}{\alpha}$. Since $\alpha$ (defined as the ratio of *free* to total electrolyte) is equal to $1 - i$, we may write $H^+ = Ki/(1 - i)$; that is to say, *free* hydrogen ion equals $Ki/(1 - i)$. This will be recognized as entirely analogous to the statement *free inhibitor equals* $Ki/(1 - i)$ (p. 562). These equalities are true for all zones, but since the curves of Fig. 2 are plotted against *total I*, they will depict the above functions *only where total I is equivalent to free I*; namely, in the limiting zone A curve, where $E' \leqq 0.1$. This single curve, then, represents the Henderson-Hasselbalch equation, and it is quite natural that its slope, 0.575, at $i = 0.5$, should be identical with the maximum buffering capacity, $\beta_M$.

reversibly associated complex should lead to dissociation. With electrolytes, for example, and with antigen-antibody complexes, the phenomenon is a familiar one. Hussey and Northrop (5), working with trypsin and the inhibitory substance contained in plasma, observed that dilution resulted in dissociation and used this as evidence for the formation of a reversible complex acting in accordance with the mass action law. However, no work has come to our attention putting the dilution effect itself on a sound quantitative basis.

In diluting an enzyme-inhibitor mixture the specific enzyme concentration $E'$ will always be changed to exactly the same degree as the specific inhibitor concentration $I'$; in other words, the ratio $I'/E'$ (or $I/E$) will be maintained constant. Thus, to represent dilution of such a mixture on Fig. 2 we travel from the original $E'$ curve to the more dilute $E'$ curve, but we must at the same time move a corresponding distance along the $I'$ axis.

We will begin by considering the effect of dilution within zone C, where $E' \geqq 100$. For example, to dilute from $E' = 1000$ to $E' = 100$, $I'$ is necessarily also diluted 10 times, so that from a selected point on the curve $E' = 1000$ we move to the left 1 log unit measured along the $I'$ axis. It will be seen that having moved this distance horizontally to the left, we find ourselves on the curve $E' = 100$ without having to move up or down, so that the inhibition $i$ remains unchanged. *The concrete meaning of this is that within zone C dilution has no effect whatever upon the fractional inhibition i.*

To represent dilution in the region where $E' \leqq 0.1$, we carry out the same steps as above; but the results are found to be quite different. For example, if we dilute 10 times in this region (*e.g.*, $E' = 0.1$ to $E' = 0.01$) we must again move 1 log unit to the left on the $I'$ axis (since $I'$ is also diluted 10 times). However, the curve for $E' = 0.01$ is practically identical with that for $E' = 0.1$, so that we must finally find ourselves on the same $E'$ curve from which we began. This necessarily involves traveling up the curve and thereby ending with a smaller fractional inhibition $i$ than we started with. Since all values of $E'$ smaller than 0.1 are represented by the same curve, *it is characteristic of dilution in zone A that the change produced in i is not influenced by initial or final specific enzyme concentrations, but only by the factor of dilution.*

If we carry out the same steps in zone B we find that our travel to the left on the $I'$ axis always carries us beyond the proper $E'$ curve, so that we are forced, as in zone A, to travel up the curve and thereby change the value of $i$. *In zone B, therefore, the fractional inhibition does change with dilution but the amount of change depends not only upon the factor of dilution, but also upon the initial and final E'.*

We have shown that in zones A and B dilution of an enzyme-inhibitor mixture results in dissociation so that the measurable inhibition $i$ is decreased; and that in zone C this does not occur. We have also pointed out that many enzyme systems operate in zone B and that others which may operate in zone

C *in vivo* are brought into zone B or even into zone A by dilution for experimental purposes. Therefore, since so many known enzyme-inhibitor systems are subject to the dilution effect, it will be necessary to place this effect upon a quantitative basis so that appropriate corrections may be made.

It would be a mistake to think that the errors arising from neglect of the dilution effect are small. As a matter of fact, they are so enormous as to invalidate conclusions based upon the application of experimental values of $i$ at various dilutions to undiluted serum or other body fluids. It is also probable that often observed discrepancies between experimentally determined values of $i$ and concomitant physiological responses may now be reconciled when the corrections for dilution are applied.

### *Practical Tests*

The magnitude of the dilution effect will be considered in a quantitative way below. We wish first to point out some useful tests based upon the zone behavior outlined above.

*1. Test for Presence of Inhibitor.*—If no inhibitor is present, there is no inhibition, regardless of dilution, and the enzyme always works at its maximum velocity. This is shown in Fig. 2 by the fact that as $I'$ approaches zero, $i$ becomes zero for all the values of $E'$. This may seem rather obvious, but it is no less important, for failure to show direct proportionality between reaction velocity and enzyme concentration (provided that diffusion is not a significant factor) is strong evidence for the presence of a reversible inhibitor. Thus, in zones A and B, if such an inhibitor is present, the reaction velocity after dilution will be greater than direct proportionality would allow.

*2. Rough Test for Zone Behavior.*—It has previously been mentioned that the slope of the experimentally determined inhibition curve at $i = 0.5$ yields a rough indication of the value of $E'$ and hence of the zone (see p. 568).

*3. Test for Zone A Behavior.*—It will be recalled from Fig. 2 that for $E' \leqq 0.1$, $i$ is determined solely by $I'$. Thus, if, and only if, a given total concentration of inhibitor produces the same inhibition at two different concentrations of enzyme, the system must be in zone A, at both enzyme concentrations.

*4. Test for Zone C Behavior.*—It was shown above that only in zone C is the dilution effect absent. Thus, if, and only if, dilution of any mixture of enzyme and inhibitor produces no change in inhibition, the system must be in zone C.

### *Algebraic Representation of the Dilution Effect*

The magnitude of the dilution effect for any values of $E'$ and $I'$, for any initial inhibition ($i$), and for any factor of dilution, may best be determined algebraically. Although the graphical method given above is useful for visualizing what is going on, it does not afford the accuracy of an algebraic treatment.

Let equation 3*B* be rewritten in the form

$$I' = \frac{i_1}{1 - i_1} + i_1 E' \tag{5}$$

where $i_1$ is the observed fractional inhibition, and

$E'$ is the concentration of enzyme in the reaction mixture where the observation is made.

Let $N$ be a factor by which both $I'$ and $E'$ must be multiplied in order to reach the concentration at which the new inhibition, $i_2$, is to be calculated. Thus $N > 1$ in going, for example, from diluted serum with an observed inhibition of $i_1$ to undiluted serum with an inhibition of $i_2$. Conversely, $N < 1$ when it is desired to calculate the inhibition, $i_2$, in a system more dilute than the reaction mixture on which the determination is made. When the reactants are at $N$ times their initial concentration, equation 3*B* becomes

$$NI' = \frac{i_2}{1 - i_2} + i_2 NE'$$

Dividing by $N$

$$I' = \frac{1}{N} \cdot \frac{i_2}{1 - i_2} + i_2 E' \tag{6}$$

eliminating $I'$ by combining equations 5 and 6,

$$\frac{1}{N} \cdot \frac{i_2}{1 - i_2} + i_2 E' = \frac{i_1}{1 - i_1} + i_1 E'$$

Solving for $i_2$,

$$i_2 = \tfrac{1}{2}\left\{\left[\frac{1}{E'} \cdot \frac{i_1}{1 - i_1} + i_1 + \frac{1}{NE'} + 1\right] - \sqrt{\left[\frac{1}{E'} \cdot \frac{i_1}{1 - i_1} + i_1 + \frac{1}{NE'} + 1\right]^2 - 4\left[\frac{1}{E'} \cdot \frac{i_1}{1 - i_1} + i_1\right]}\right\}$$

and simplifying[8] the term under the radical,

$$i_2 = \frac{1}{2}\left\{\left[\frac{1}{E'} \cdot \frac{i_1}{1 - i_1} + i_1 + \frac{1}{NE'} + 1\right] - \sqrt{\left[\frac{1}{E'} \cdot \frac{i_1}{1 - i_1} + i_1 + \frac{1}{NE'} - 1\right]^2 + \frac{4}{NE'}}\right\} \tag{7B}$$

[8] Let

$$\left[\frac{1}{E'} \cdot \frac{i_1}{1 - i_1} + i_1\right] = X \quad \text{and} \quad \left[\frac{1}{NE'}\right] = Y$$

Then

$$[X + Y + 1]^2 - 4X = [X + Y - 1]^2 + 4Y.$$

Equation $7B$ is a general solution for the inhibition at a new dilution when the original $E'$, the original inhibition ($i_1$), and the dilution factor are known. Because it involves the difference of two terms of the same magnitude, calculation from this equation must be accurate to three decimal places, but otherwise the equation is not especially cumbersome.

It is of some interest to examine the limiting cases in this equation. It will be evident that when $i_1 = 0$, $i_2 = 0$. When $E' \doteq \infty$, $i_2 = i_1$ ($7C$). Both these results have been previously derived in this paper.

When $E' \doteq 0$, evaluation of the equation becomes very difficult because the terms containing $E'$ become infinite. However, the same steps used to derive equation $7B$ from equation $3B$ can be applied to equation $3A$, which describes the zone where $E' \doteq 0$. It is then found that

$$i_2 = \frac{Ni_1}{1 - i_1 + Ni_1} \qquad (7A)$$

This equation is valid for all systems within zone A and may be applied in place of the more complex equation $7B$.

### *Plot of Dilution Effect*

A practical way of visualizing the dilution effect quantitatively is presented in Fig. 3. This figure is simply a plot based upon equation $7B$, a value of $E'$ being used which corresponds with our experimentally determined specific concentration of horse serum cholinesterase (see p. 578). This particular system was found to operate in zone $B$, $E'$ being equal to 3.29 in undiluted serum. Arbitrarily selecting a number of values of inhibition in 22.2 per cent serum, corresponding values of $i_2$ were calculated for each of several dilutions. The 22.2 per cent serum inhibitions were then represented as a straight line with slope = 1, and the various corresponding values of $i_2$ plotted accordingly as abscissae.

To use this graph for dilution or concentration one simply travels to right or left on a horizontal line. The values of inhibition are read off directly from the abscissa. Thus, for example, an inhibition $i = 0.8$ in serum becomes 0.5 at 22.2 per cent, 0.2 at 4.54 per cent, and 0.05 at 1.0 per cent. In this example the absurdity of concluding from a determination in 1.0 per cent serum that the undiluted serum was practically uninhibited needs no further emphasis.

It is perhaps best to think of the dilution effect in terms of the distortion of *ranges* of inhibition. To take the most extreme example, reference to Fig. 3 will show that the entire range from 0.1 to 0.9 in actual serum is represented at 1.0 per cent by the experimentally determined range 0.01 to 0.1. On the other hand, the whole experimental range 0.1 to 1.0 at this dilution is seen to represent the very small range 0.9 to 1.0 in actual serum. Similar but less serious distortions are observed at higher experimental concentrations.

These considerations make it plain that methods involving considerable dilution are less useful the greater the factor of dilution. In the example cited, for observed values of $i$ between 0.01 and 0.1 experimental errors are magnified

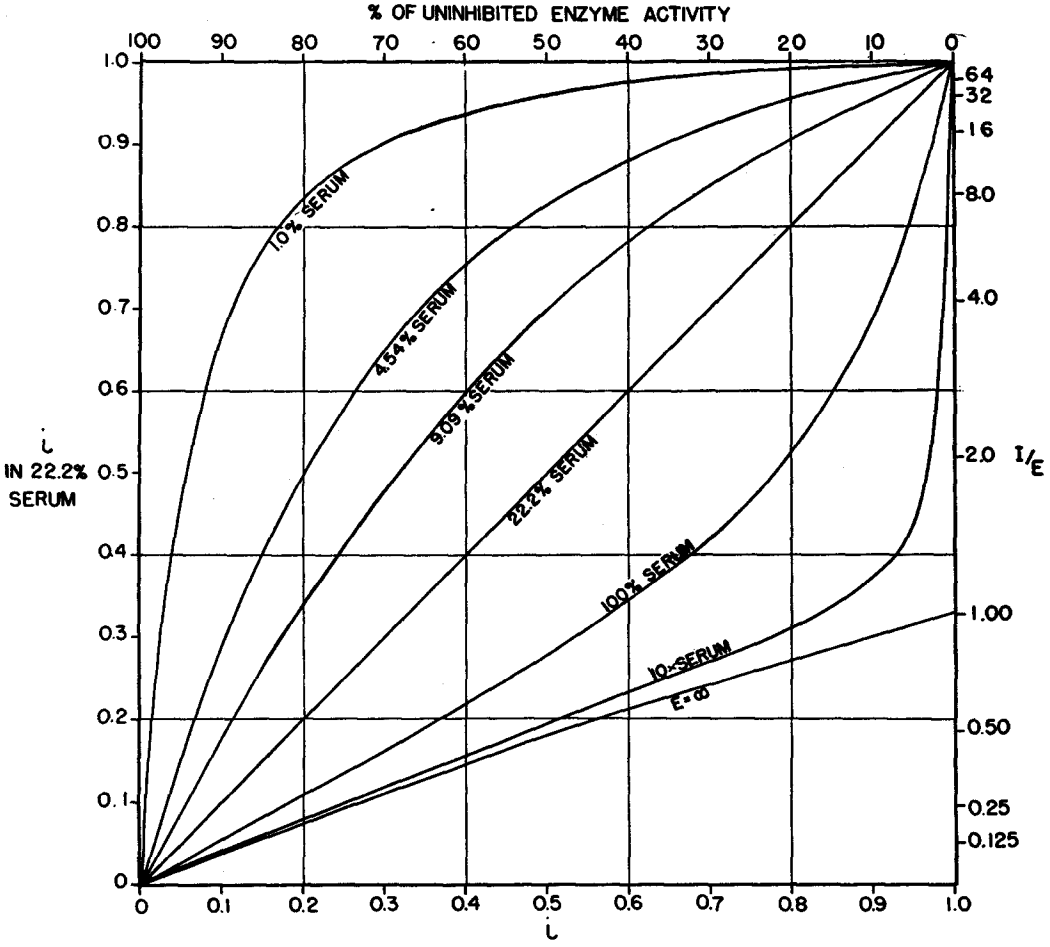


FIG. 3. Abscissa, fractional inhibitions $i$ at various concentrations. Ordinate, left, corresponding inhibitions in 22.2 per cent serum.

Ordinate, right, ratios of total inhibitor to total enzyme concentrations. This ratio remains constant with dilution of any particular enzyme-inhibitor mixture.

tenfold when the appropriate conversion to serum inhibition is performed. At the same time, because observed values of $i$ between 0.1 and 1.0 represent so small and comparatively unimportant a range of actual serum inhibitions, the major part of the method's usefulness is wasted.

The chief theoretical considerations and practical tests implicit in the concept of zone behavior of enzyme systems are summarized in Table I.

TABLE I

| Zone A | Zone B | Zone C | Remarks |
|---|---|---|---|
| $E' < 0.1$ | $0.1 < E' < 100$ | $E' > 100$ | Values given are approximations where $\Delta i = 0.01$. For exact boundaries as a function of $i$, see Fig. 1 |
| $I = K\frac{i}{1-i}$ (2A)<br>Total = free<br>$I' = \frac{i}{1-i}$ (3A) | $I = K\frac{i}{1-i} + iE$ (2B)<br>Total = free + bound<br>$I' = \frac{i}{1-i} + iE'$ (3B) | $I = iE$ (2C)<br>Total = bound<br>$I' = iE'$ (3C) | $E$ does not enter equations for zone A, nor $K$ in those for zone C. In zone C, inhibitor combines quantitatively with enzyme; true for all values of $i$ reasonably below 1.0 |
| $i$ produced by a given $I'$ is independent of $E'$ | $i$ is dependent on both $I'$ and $E'$ | $i$ is dependent on both $I'$ and $E'$. $I'$ required to produce a given $i$ is directly proportional to $E'$ | Can be used as criteria for zones A and C |
| Because $E$ does not appear in equations, no definite value can be assigned to it by any method involving measurement only of reaction velocities and of $I$. $K$ can be evaluated; $E'$ cannot | Both $E$ and $K$ appear and can be assigned definite values; so can $E'$ | Because $K$ does not appear in equations, it cannot be evaluated by any method involving only reaction velocities and $I$. $E$ can be evaluated; $E'$ cannot | The use of the terms $I'$ and $E'$ in equation 3C does not permit evaluation of $K$, since $K$ cancels out of both sides of the equation |
| Michaelis equation applies | Michaelis equation does not apply | Michaelis equation does not apply | Error in the determination of $K$ by measuring the concentration of $I$ when $i = 0.50$ rapidly becomes great when $E'$ exceeds 0.1 |
| $\frac{di}{d\log I'} = 0.575$, when $i = 0.50$ for all values of $i$ | $0.575 < \frac{di}{d\log I'} < 1.151$, when $i = 0.50$ for all values of $i$ | $\frac{di}{d\log I'} = 1.151$, when $i = 0.50$ for all values of $i$ | Can be used as criteria for zones and for rough evaluation of $E'$ in zone B |
| Dilution effect present and independent of $E'$<br>$i_2 = \frac{Ni_1}{1 - i_1 + Ni_1}$ (7A) | Dilution effect present and varies with $E'$<br>$i_2 = f(i_1, E', \text{and } N)$ see equation 7B | Dilution effect absent: $i$ does not change on dilution<br>$i_2 = i_1$ (7C) | Can be used as a criterion of zone C |

## EXPERIMENTAL

To test the validity of any hypothesis it is sufficient to test any one function that includes all the assumptions implicit in the original hypothesis. The

dilution equation provides such a test, and will be shown to describe the behavior of mixtures of physostigmine and cholinesterase with satisfactory accuracy over a wide range of enzyme concentration.

*Determination of E'*

(*a*) *Method.*—Determinations of the cholinesterase activity of unpurified horse and dog serum for the calculation of the dissociation constant of the enzyme-inhibitor complex and the molar concentration of enzyme centers were done by the method of Friend and Krayer (6). The final reaction mixture contains 22.2 per cent serum and 2.7 per cent (0.12 molar) acetylcholine bromide in bicarbonate Ringer solution at pH 7.4 and 38°C., equilibrated continuously with vigorous mechanical stirring against a 5 per cent carbon dioxide —95 per cent nitrogen gas mixture. An equimolecular amount of carbon dioxide is displaced by the production of acetic acid from acetylcholine during its hydrolysis by the enzyme. Exactly 1.00 cc. of serum is added to 3.0 cc. of bicarbonate Ringer and equilibrated for 15 minutes; after 0.50 cc. of 24 per cent acetylcholine bromide in Ringer solution is added and equilibration continued for 3 minutes more, a 1 cc. aliquot is removed, and another aliquot is removed exactly 20 minutes later. The difference in the carbon dioxide content per liter of the two samples done by the Van Slyke manometric method equals the millimoles of acetylcholine hydrolyzed per liter of reaction mixture per 20 minutes.

Expressed in terms of millimoles of acetylcholine hydrolyzed per liter of 100 per cent serum per hour, the average titer of horse serum by this method was 200 mM/liter/hr., with a range of 160 to 240 in samples from different animals. The average value for dog serum was 120 mM/liter/hr., with a range of 70 to 160. In the work with horse serum described below, a pooled batch of sterile serum with a titer of 202 mM/liter/hr. was used. This value was found to remain constant for a number of weeks even in serum held at 38°C. without sterile precautions.

Measurement of the fractional inhibition produced by a known concentration of physostigmine salicylate was carried out by dissolving a known amount of drug in the 3.00 cc. of bicarbonate Ringer solution used to dilute the serum before equilibration. The inhibitor was thus in contact with the enzyme for 18 minutes before the start of the 20 minute period of measurement. This order of adding the reactants is important inasmuch as consistently smaller values of $i$ are obtained if the inhibitor is added *after* the substrate. (See discussion on competition, p. 583.)

(*b*) *Results.*—Experimental points for the fractional inhibition produced in 22.2 per cent horse serum by various molar concentrations of physostigmine in the reaction mixture are plotted in Fig. 4. These define the whole curve of inhibition, $i$, *versus* the logarithm of the molar concentration of inhibitor, $I$,

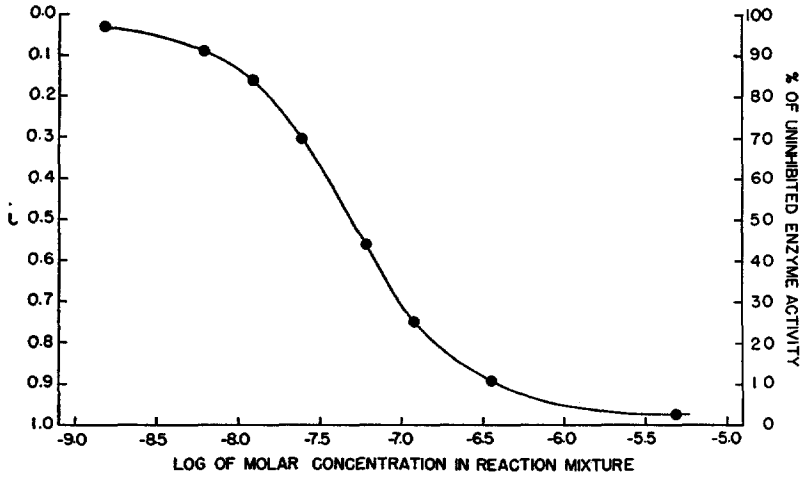


FIG. 4. Fractional inhibition of horse serum cholinesterase as a function of physostigmine salicylate concentration. Enzyme concentration in reaction mixture, $E = 2.7 \times 10^{-8}$ molar, and $E' = 0.73$ (see Fig. 5). This is a specific example of the generalized curves depicted in Fig. 2.

TABLE II

| A | B | C | D | E |
|---|---|---|---|---|
| $i$ | Observed $I \times 10^8$ (molar) | $\log_{10} I$ (molar) | $I/i \times 10^8$ | $1/(1-i)$ |
| *0.03 | *0.15 | −8.82 | 5.0 | 1.03 |
| 0.05 | 0.32 | −8.50 | 6.4 | 1.05 |
| *0.09 | *0.62 | −8.21 | 6.9 | 1.10 |
| *0.16 | *1.23 | −7.91 | 7.7 | 1.19 |
| 0.20 | 1.59 | −7.80 | 7.9 | 1.25 |
| *0.30 | *2.45 | −7.61 | 8.2 | 1.43 |
| 0.40 | 3.63 | −7.44 | 9.1 | 1.66 |
| 0.50 | 5.12 | −7.29 | 10.2 | 2.00 |
| *0.56 | *6.03 | −7.22 | 10.8 | 2.28 |
| 0.60 | 7.25 | −7.14 | 12.1 | 2.50 |
| 0.70 | 10.0 | −7.00 | 14.3 | 3.33 |
| *0.75 | *12.2 | −6.92 | 16.3 | 4.00 |
| 0.80 | 17.0 | −6.77 | 21.2 | 5.00 |
| *0.89 | *36.3 | −6.44 | 40.7 | 9.10 |
| *0.97 | *490 | −5.31 | 505 | 33.3 |

* Indicates observed values; other values of $i$ and $I$ interpolated from plot of observed values of $i$ and $I$ (Fig. 4).

within very close limits, and the values are typical of other runs. The observed values and additional points interpolated graphically from Fig. 4 are tabulated in columns A, B, and C of Table II.

From the values of log $I$ where $i = 0.3$ and 0.7, the slope $di/d \log I$, in this segment is found to equal 0.66. Since this value lies between the limits 0.575 and 1.151, the function is at least compatible with reaction B (see p. 568 above). Substituting the value $di/d \log I = 0.66$ in equation 4,[9] the preliminary figure for $E'$ turns out to be approximately 0.7. This indicates that the reaction mixture probably lies in the lower half of zone B. A more rigorous method for determining $K$ and $E$, and hence $E'$, is therefore in order. The method had been applied to cholinesterase and prostigmine by Easson and Stedman (7).

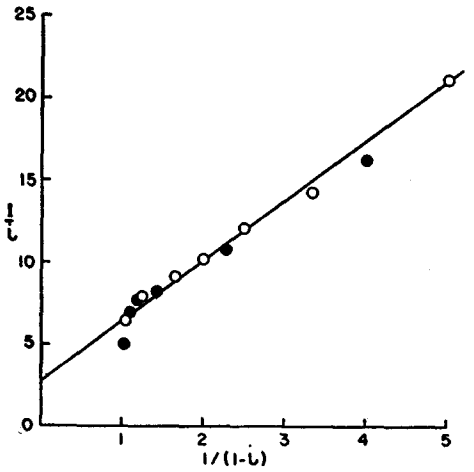


FIG. 5. Graphic method of determining $K$ and $E$ (molar) for serum cholinesterase-physostigmine system by plotting $I/i \times 10^8$ against $1/(1 - i)$. Values are tabulated in Table I. $E$ = ordinate intercept of the straight line = $2.7 \times 10^{-8}$. $K$ = slope of the straight line = $3.7 \times 10^{-8}$. ● = observed values. ○ = interpolated values.

Let equation $2B$ be divided by $i$. Then,

$$\frac{I}{i} = K \cdot \frac{1}{1 - i} + E \tag{8}$$

This equation is linear with respect to $I/i$ and $1/(1 - i)$. A plot having these terms as ordinate and abscissa respectively will therefore yield a straight line if the observed values are compatible with the assumptions upon which the equation is based; the slope will numerically equal $K$, and the ordinal intercept will equal $E$. Calculated values of $I/i$ and $1/(1 - i)$ appear in columns D and E of Table II and are plotted in Fig. 5.

Since $i$ and $(1 - i)$ appear as the denominators of these two terms, a small absolute error in $i$ will have the greatest numerical effect on $I/i$ when $i \doteq 0$,

[9] Equation 4 defines the term $di/d \log I'$. However, since $I$ differs from $I'$ by a constant $K$, the expression for slope used here will be equal to the term in equation 4.

and on $1/(1 - i)$ when $i \doteq 1$. For these numerical reasons, and because experimental accuracy of the determination of $i$ also falls off at the extremes, this method is most practical for values of $i$ between 0.2 and 0.8.

The points in Fig. 5 are seen to lie on a straight line, from the slope and intercept of which it is found that $K = 3.7 \times 10^{-8}$ and $E = 2.7 \times 10^{-8}$ molar, so that $E' \equiv E/K = 0.73$. This value agrees with the estimate obtained by use of equation 4. It is, however, a more rigorous test of the compatibility of the data with equation $2B$ since it embraces a larger segment of the whole curve. The value of $E'$ obtained by this method places the system cholinesterase-physostigmine in horse serum within zone B ($E' = 3.29$ in undiluted serum). We should therefore expect on theoretical grounds that the dilution effect ought to be demonstrable in this system. The actual correspondence between theory and experiment may now be presented.

### *Dilution Effect*

(*a*) *Method.*—For determination of the effect of diluting various mixtures of enzyme and inhibitor, the Warburg apparatus was used (method of Ammon (8)), since this method permits measurement of a wide range of reaction velocities. The temperature, pH, order of addition of reactants, timing, and concentrations were substantially the same as with the method of Friend and Krayer. Satisfactory agreement is obtained with uninhibited serum by the two methods when reduced to terms of millimoles acetylcholine hydrolyzed per liter of 100 per cent serum per hour. Measurements were made over the period from 3 to 23 minutes after addition of substrate, except in the case of 22.2 per cent serum (reaction mixture concentration) when, because of the high reaction velocity, 3 and 13 minute readings had to be used.

(*b*) *Results.*—In the absence of inhibitor the velocity of acetylcholine hydrolysis at various dilutions was approximately proportional to the serum concentration. A slight tendency for the velocity to increase relative to serum concentration was noted at the greatest dilutions, but this was probably on a basis of less $CO_2$ retention than at greater concentrations of serum.

The experimental results are summarized in Table III. Each horizontal row represents the inhibition in a single enzyme-inhibitor mixture, determined at four different dilutions (*i.e.*, $I/E$ held constant). Observed values are in bold-face type, and for each such value are calculated (from equation $7B$) the corresponding points at every other dilution and in undiluted serum. Thus in each horizontal line are found one observed value and four corresponding values.

For a given mixture at a particular dilution the observed and calculated values are seen to agree quite satisfactorily, with two or three exceptions at the extremes of dilution and inhibition. The best set of values is that for $I/E = 10.7$, where the inhibition ($i$) varies from 0.85 to 0.19 if the mixture is diluted

from 22.2 per cent to 1.0 per cent of its initial concentration, while in undiluted serum as it would exist in the experimental animal, the inhibition is 0.96.

TABLE III

*Effect of Dilution on Inhibition, i*

$E'$ in undiluted serum = 3.29

| Serum concentration in per cent of undiluted serum | 1.00 | 4.54 | 9.09 | 22.2 | 100 |
|---|---|---|---|---|---|
| *I/E* | | | | | |
| | **0.020** | 0.08 | 0.14 | 0.24 | 0.43 |
| 1.1 | 0.020 | **0.07** | 0.12 | 0.21 | 0.39 |
| | 0.015 | 0.06 | **0.10** | 0.18 | 0.33 |
| | 0.025 | 0.10 | 0.17 | **0.30** | 0.53 |
| 1.8 | | **0.09** | | 0.24 | 0.43 |
| | | 0.11 | | **0.30** | 0.53 |
| | **0.02** | 0.08 | 0.14 | 0.24 | 0.43 |
| 2.1 | 0.05 | **0.18** | 0.28 | 0.46 | 0.74 |
| | 0.05 | 0.17 | **0.28** | 0.46 | 0.74 |
| | 0.05 | 0.19 | 0.30 | **0.48** | 0.76 |
| 9.1 | | **0.36** | | 0.72 | 0.91 |
| | | 0.33 | | **0.69** | 0.90 |
| | **0.19** | 0.50 | 0.67 | 0.83 | 0.96 |
| 10.7 | 0.21 | **0.54** | 0.70 | 0.85 | 0.96 |
| | 0.20 | 0.52 | **0.68** | 0.84 | 0.96 |
| | 0.21 | 0.54 | 0.70 | **0.85** | 0.96 |
| | **0.22** | 0.55 | 0.71 | 0.86 | 0.95 |
| 21.5 | 0.37 | **0.72** | 0.84 | 0.93 | 0.98 |
| | 0.30 | 0.64 | **0.79** | 0.90 | 0.97 |
| | 0.30 | 0.64 | 0.79 | **0.90** | 0.97 |
| | **0.76** | 0.92 | 0.97 | 0.99 | 0.998 |
| 215 | 0.56 | **0.86** | 0.94 | 0.97 | 0.995 |
| | 0.50 | 0.82 | **0.90** | 0.96 | 0.99 |
| | 0.50 | 0.82 | 0.90 | **0.96** | 0.99 |

Although these experimental data do not constitute a perfect verification of our theoretical premises, we believe that they are sufficiently impressive to serve as strong corroborative support for the validity of equation $7B$, and consequently of the zone concept in general. Apart from all theoretical considerations, the practical corollary of equation $7B$ has been adequately proven: that it is unwarranted to assume that determinations of the state of an enzyme-

inhibitor system *in vitro* give an accurate picture of the state of that system in the circulating serum. It is now possible for the first time to calculate these serum enzyme inhibitions *in vivo* and furthermore to compare the results of different investigators, who may use various methods involving a variety of dilutions of the enzyme-inhibitor mixture.

### *Turnover Number*

Since under the experimental conditions described, 1 liter of uninhibited reaction mixture hydrolyzes 15 millimoles of acetylcholine in 20 minutes, and since $E = 2.7 \times 10^{-8}$ molar, it follows that each active enzyme center breaks down 450 molecules of acetylcholine per second. This turnover number is $\frac{1}{3}$ that reported by Easson and Stedman (7), and our dissociation constant is 3 to 4 times that reported by Roepke (9) working on serum cholinesterase largely freed of inert protein by the method of Stedman and Stedman (10). Roepke, moreover, noted that 3 to 4 times as much physostigmine was necessary to produce a given inhibition in crude serum as in the purified product, probably because of fixation of inhibitor by inert protein.

Inspection of equation 2 *B* shows that a false high value of $I$, resulting from such a circumstance, would raise the apparent value of $E$, and account for the direction of divergence in our figure for the turnover number. Nevertheless, we wish to emphasize that our values of $K$ and $E$, and the conjugate values of $i$ and $I$ so obtained, fit the results within the limits of experimental error; and that the values obtained with purified enzyme are inapplicable to crude serum, which, after all, is what circulates in the vessels of the experimental animal.

## DISCUSSION

### *Evolution of the Concept of Zone Behavior*

Since there is nothing in the foregoing analysis of the union of one enzyme center with one molecule of substrate or inhibitor that is not implicit in the law of mass action, the question arises why the differences in zonal behavior and the dilution effect have not previously been pointed out and put to use. The answer perhaps lies in the formulation itself. It has been shown that the enzyme systems that have most frequently been used as prototypes for general discussions of enzyme kinetics ordinarily behave like monomolecular reactions (are pseudomonomolecular) under the conditions of measurement; *i.e.*, they lie in zone A. The cholinesterase-physostigmine system used as a prototype in this paper was examined at enzyme concentrations that place the system in a zone where the monomolecular function no longer adequately describes the results obtained. Easson and Stedman (7), recognizing this fact in their paper on the kinetics of a similar system, correctly used the full second order function but did not carry the analysis to its ultimate conclusion.

Although a first order kinetic function has the advantage of simplicity, its application to a true second order reaction such as the reversible union of an

enzyme and its inhibitor may lead to serious error under conditions where the concentration of the second reactant becomes of importance. It therefore seemed sounder to us to set up the function covering the reversible union of two reactants to form a complex, and then to attempt to establish rigorous limits within which the use of the monomolecular function causes less than some definite and negligible error. It has become apparent in the course of this analysis that such systems show not two, but three zones of behavior: a zone adequately described by the monomolecular function (zone A), a zone where the full bimolecular function must be used (zone B), and finally a zone in which the reactants will appear to combine with each other stoichiometrically according to the law of definite proportions, although the reaction is still fully reversible (zone C).

### *Biological Significance of Zone C Behavior*

The likelihood that many enzymes, at the points where they function in tissue, are highly concentrated and therefore in zone C with respect to their substrates or to inhibiting substances has already been mentioned. It follows from the stoichiometric behavior of the reactants in this zone that if a biological effect is found to be a linear function of the dose or concentration of an inhibitor, one need not necessarily conclude that the reaction is irreversible, but only that, if reversible, the reactant with which the inhibitor combines has a specific concentration high enough to place the system in zone C.

There is another conclusion which should prove to have widespread practical application in experimental pharmacology. Since in zone C inhibition ($i$) is equal to the ratio $I/E$ without reference to $K$, and since it is precisely the dissociation constant $K$ that distinguishes one inhibitor from another in the effect upon a given enzyme, it follows that for any enzyme in tissue at fairly high concentration, *all reversible inhibitors should produce the identical effect*, provided only that the various values of $K$ are all of such magnitude that the system remains in zone C. A simple example will illustrate the point. Let us consider, as Nachmansohn (11) has shown, that cholinesterase in the body is very highly concentrated at the motor end-plates; for example, $E = 10^{-4}$. We will now assume two inhibitors, one, like physostigmine, with $K$ about $10^{-8}$, the other with $K$ about $10^{-6}$. Having determined the dissociation constants in dilute serum, and having observed the greater potency of physostigmine under such conditions, we would naturally assume that the biological response to this drug would be far greater, perhaps a hundred times as great. We now see, on the contrary, that since both values of $K$ are such as to leave the system within zone C there should be no demonstrable difference in the biological action of the two drugs. If this prediction is sound, we must conclude that with drugs of this type it is futile to seek increased potency except as such efforts are directed toward the problems of toxicity, distribution, inactivation, excretion, etc.

*Definition of Zone Boundaries in Terms of I*

Since reaction $B$, denoting the reversible union of two reactants, is symmetrical with respect to both reactants, the question arises why the specific enzyme concentration, $E'$, rather than the specific inhibitor concentration, $I'$, has been used to define the zone boundaries. The answer lies in the fact that the choice of variables in the function relating the concentrations of the reactants and their complex (equation $3B$), is determined by what quantities are conveniently measurable and what quantities it is desirable to calculate by means of these equations. It is usually convenient to measure the total concentration of inhibitor employed, and it seems logical to employ a term to denote the total concentration of enzyme, $E$. Since in most enzyme work the fraction of total enzyme, $i$, that is in the combined form is of primary interest, it is both logical and convenient to introduce this asymmetrical term into the kinetic equations. However, under circumstances where interest was focused upon the fraction of total substrate or inhibitor that is combined with enzyme, there would be equal justification for exchanging the places of $E$ and $I$ and letting $i$ now represent $I_{\text{combined}}/I_{\text{total}}$. The zone boundaries would then be determined by the specific inhibitor concentration, $I'$, whose numerical boundary values will be the same as those for $E'$. The application of this concept to an analysis of the rate of destruction of acetylcholine at the nerve ending in relation to the refractory period of the nerve will be treated in a note to be published later.

*Extension of the Analysis to More Complex Systems*

It has been emphasized that this analysis can be applied to any reaction of the form $A + B \rightleftharpoons AB$. This would include many antigen-antibody reactions, weak acid or base dissociations, solubility products, etc., as well as certain enzyme systems.

In considering the case of an enzyme combining with substrate alone, we must, of course, make the customary "steady state" assumptions for reaction $A$ ($E + X \rightleftharpoons EX \rightarrow E + Sp$), the concentration of $EX$ remaining constant and that of $X$ not changing appreciably during the reaction. The *combined form* of the enzyme is now *active*, so that we may call $(ES)/E$ the *fractional activity*, and designate it by $a$, which will then be substituted for $i$ in all the equations. With this minor reorientation, the analysis applies to uninhibited enzyme reactions, which should display the same zone behavior and dilution effects already demonstrated for inhibited systems.

For reactions of a higher kinetic order, it will be desired to generalize the foregoing analysis to apply to the case where one molecule of a reactant combines reversibly with $n$ molecules of a second reactant ($E + nI \rightleftharpoons EI_n$). It can be shown by the same steps used to derive equation $3B$ that

$$I' = \sqrt[n]{\frac{i}{1-i}} + niE'$$

where $E' = E/\sqrt[n]{K}$, and $I' = I/\sqrt[n]{K}$. This is merely a more generalized form of equation $3B$, and the same analysis can be applied to derive zone boundaries and the equations describing the dilution effect. All statements applying to the zones will still apply. In general, as $n$ increases the boundaries of zone B will approach each other. Equation $7A$ describing the dilution effect in zone A becomes

$$i_2 = \frac{N^n i_1}{1 - i_1 + N^n i_1}$$

An increase in $n$ very markedly increases the change of inhibition with dilution, an effect that can be experimentally measured. This function thus provides a very sensitive criterion for testing the number of molecules of inhibitor, for instance, that combine to form an inactive complex.

### *Limitations of This Analysis*

The analysis developed in this paper is incomplete in at least one important respect, as a consequence of which important limitations are placed upon some of our conclusions. As already mentioned at the outset, the assumption of a reversible reaction between enzyme and inhibitor makes it mandatory also to assume that the addition of substrate for purposes of determination results in the displacement of a certain number of inhibitor molecules from combination, provided only that inhibitor and substrate combine with the same active center of the enzyme molecule. Thus substrate addition must cause a decrease in $i$. That the effect can be noticed even within the initial 20 minute period required for the determination was indicated on p. 575. We have pointed out that the quantitative significance of this competition effect will depend upon the constants for any particular system. A completely valid treatment, however, should be based upon the final equilibrium attained between enzyme, inhibitor, and substrate, rather than upon the first two alone. More accurate values of $K$ and $E$ could then be obtained, for if competition is significant within the first 20 minutes the curve of Fig. 4 may no longer be interpreted on the basis of reaction B and equations $2B$ and $3B$. Work placing the competition effect on a sound theoretical and experimental basis is now in progress in this Laboratory and will be the subject of a subsequent publication.

Despite its inadequacies, the present *non-competitive* treatment nevertheless applies *fully*: (1) to the case of an inhibitor which reversibly inactivates an enzyme by combining at a different point from the substrate, or by causing physical alteration of the enzyme molecule; (2) to the case of uninhibited enzyme-substrate and other comparable systems, provided the substrate concentration does not change appreciably during the course of the reaction. The present treatment applies *practically* to the case where competition is not significant compared with the function being measured. It is on this basis that

we feel justified in illustrating our method and confirming the dilution effect by means of the cholinesterase-physostigmine system, which has in the past been considered practically non-competitive.

SUMMARY

1. The kinetics of the reversible combination of one enzyme center with one molecule of a substrate or inhibitor is treated as a true bimolecular instead of a pseudomonomolecular reaction. The general equations describing such a reaction are presented and analyzed algebraically and graphically.

2. A new term, "specific concentration," is introduced to denote the concentration of reactants in units equal to the dissociation constant. Its use makes the kinetic equations universally applicable to all reversible systems of the given type.

3. It is shown that such a system exhibits three "zones" of behavior. Each zone is characterized and shown to exhibit significant differences in the function relating the concentrations of the components of the system at equilibrium. The zone boundaries are rigorously defined in terms of the specific enzyme concentration, for the mathematical error tolerable with a given experimental accuracy; and approximate boundaries for practical use are proposed.

4. The classical treatment of enzyme kinetics is shown to be a limiting case valid only for low specific enzyme concentrations (zone A) and to be inapplicable in a number of systems whose dissociation constants are very small or whose molar enzyme concentrations are very great, and in which, therefore, the specific enzyme concentrations are large. See Table I for a summary of zone differences.

5. In an enzyme system containing substrate or inhibitor, dilution before determination of reaction velocities is shown to be a crucial operation, entailing large changes in the fraction of enzyme in the form of a complex. The changes in fractional activity or inhibition with dilution are shown to be a function of specific enzyme concentration, the dilution factor, and the fraction of enzyme initially in the form of complex. Equations are given permitting the calculation of the state of the system at any concentration. The errors introduced into physiological work by failure to take the dilution effect into account are pointed out.

6. Experimental data are presented showing that the system composed of serum cholinesterase and physostigmine behaves as predicted by the dilution effect equations.

7. Two other conclusions of practical pharmacological importance are drawn from the theory of zone behavior:

(a) The finding that a biological response is a linear function of the dose of a drug does not necessarily mean that the reaction is irreversible, but only that if reversible, the reactant with which the drug combines has a high specific concentration.

(b) If a tissue enzyme has a high specific concentration, *all* reversible inhibitors will be equally potent in combining with it, regardless of their relative potency in dilute systems; provided only that their dissociation constants are within certain broad limits.

8. It is shown how the type of analysis here applied to bimolecular reactions can be applied *in toto* to systems of the type $E + nX \rightleftharpoons EX_n$, where $n$ molecules of substrate or inhibitor unite with one enzyme center. The zone boundaries and the magnitude of the dilution effect change with $n$, but the general characteristics of the zones are the same for all values of $n$.

9. Since the analysis is based only on mass law assumptions, it is applicable to any system that is formally analogous to the one here treated.

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