# Informations - Informationen - Informazioni - Notes

## EXPLICATIONES

### A Relationship between the Rate of Attaining Equilibrium and the Velocity Constant of the Reverse Reaction in certain Enzyme-Inhibitor Systems

The purpose of this communication is to point out a relationship based upon the law of mass action, which is applicable to a certain class of reversible reactions, yet is not apparent superficially and has not been formally stated heretofore.

It has recently been shown[1] that reversible cholinesterase inhibitors of a certain structure fall into a distinct group characterized by an unusually slow rate of reaction with the enzyme. The development of inhibition is slow (as first demonstrated by EASSON and STEDMAN[2]) and dissociation on dilution or on addition of substrate is equally slow. These observations raised the question whether there was any necessary parallelism between the rates of association and dissociation, and this led to a more general inquiry into the factors influencing the rate of attaining equilibrium in an enzyme-inhibitor system studied in the customary way.

Two peculiarities are encountered in the comparative study of reversible enzyme inhibitors. *First*, the investigator has little freedom in choosing their concentrations. If an effect is to be observed at all, one is forced (for reasons of accuracy) to measure *moderate* degrees of inhibition, i.e. to work somewhere in the middle portion of the inhibition curve. The available concentration range for any inhibitor will therefore depend upon its affinity for the enzyme; and as affinities may vary over many orders of magnitude, various inhibitors will commonly be employed at widely different concentrations. *Second*, what one measures is not the number of moles of a reaction product but rather the combination with inhibitor of a certain *fraction* of enzyme molecules. As a consequence of these two peculiar conditions, in systems of a certain type (defined below), a special physical significance can be attached to the rate of development of inhibition. Curiously, the whole course of the approach to equilibrium becomes dependent upon the velocity constant of *dissociation*.

For reversible inhibition, assuming one inhibitor molecule combines with a single enzyme center,

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

At equilibrium, *provided most inhibitor molecules remain free*,

$$\frac{(E_f)\,(I_f)}{(EI)} = \frac{k_2}{k_1} = K,$$

$$i = \frac{(EI)}{E_t}$$

$$I_t = I_f = K\,\frac{(EI)}{E_t - (EI)} = \frac{k_2}{k_1} \cdot \frac{i_e}{1 - i_e},$$

where $i$ and $i_e$ represent, respectively, the fractional inhibition in general, and at equilibrium; and $E_f$, $I_f$ denote *free* and $E_t$, $I_t$ *total* enzyme and inhibitor concentrations.

The condition that $I_f = I_t$, namely that the enzyme does not bind an appreciable fraction of the inhibitor, is essential to all that follows. It applies in any case where the Michaelis constant, $K$, can be taken equal to the inhibitor concentration producing 50% inhibition. Such systems are defined by the ratio $E_t/K$, as pointed out elsewhere (zone *A* of STRAUS and GOLDSTEIN[1]), and are exceedingly common.

Now for the rate of development of inhibition,

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

$$\frac{di}{dt} = k_1(I_t)\,(1 - i) - k_2\, i.$$

For $I_t$ we now substitute its equilibrium value and simplify, to give

$$\frac{di}{dt} = k_2 \left(\frac{1}{1 - i_e}\right)\left(i_e - i\right). \qquad \text{(Equation 1)}$$

Integrating to obtain $i$ as a function of $t$,

$$i = i_e \left\{1 - e^{-\frac{k_2 t}{1 - i_e}}\right\}. \qquad \text{(Equation 2)}$$

It follows from *Equations 1* and *2* that, under the conditions described, the course of development of a given degree of inhibition with various inhibitors acting on the same or different enzymes is a function of $k_2$, the velocity constant of *dissociation*. The particular values of the forward velocity constant, the MICHAELIS constant (or inhibitor concentration), and the enzyme concentration are of no importance. If the rates of combination of a number of inhibitors with the same enzyme are considered, and if the enzyme concentration is not varied, then $k_2$ determines the *absolute molar reaction rate* $[d(EI)/dt]$ since any given degree of inhibition always reflects the same number of combined inhibitor molecules. If different concentrations of the same or different enzymes are allowed, then $k_2$ no longer determines the absolute reaction rate, but it still determines the *rate of attaining equilibrium* $(di/dt)$. In the special case that 50% inhibition is being approached at equilibrium, the *initial* rate of development of inhibition is simply equal to $k_2$.

*Figure 1* shows *Equation 2* plotted for four values of $k_2$, when 50% inhibition is being approached at equilibrium. This kind of curve, obtained experimentally, would give $k_2$ directly even though no other information about the system were available[2]. It would only have to be shown that most of the inhibitor was free at equilibrium, which might be done either through a direct experimental approach or by measuring the slope of the equilibrium log concentration-inhibition curve, as de-

[1] A. GOLDSTEIN, Arch. Biochem. Biophys. *34*, 169 (1951).

[2] L. H. EASSON and E. STEDMAN, Proc. Roy. Soc. London [B] *121*, 142 (1936).

[1] O. H. STRAUS and A. GOLDSTEIN, J. Gen. Physiol. *26*, 559 (1943).

[2] In studying a new system one would first find a suitable inhibitor concentration, one that will produce a moderate degree of inhibition at equilibrium. Using this concentration, the time course would next be plotted, as in Figure 1. A simple procedure then would be to measure the time required to reach half of the equilibrium value and substitute this into the expression $k_2 = 0{\cdot}69\,(1 - i_e)\,/\,t$, derived from Equation 2.

scribed elsewhere[1]. In this way one might, at least in principle, determine the velocity constant of dissociation in a reaction between two unidentified components without knowing the concentration of either.

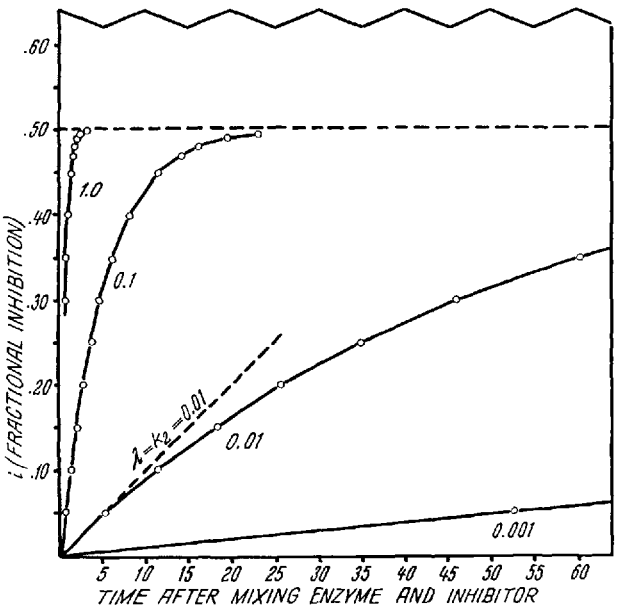


Fig. 1.–*The course of development of 50% inhibition, for different values of* $k_2$. The curves are plotted according to Equation 2. The value of $k_2$ is indicated next to each curve. The dimension of $k_2$ is *time*$^{-1}$ and its units are the same as those of the time scale. The broken line marked $\lambda$ shows the initial slope of a curve, which is equal to $k_2$.

The foregoing conclusions may sound absurd because they seem to contradict the obvious fact that the rate of any reaction is determined by the concentrations of the reacting substances and by *both* (forward and reverse) velocity constants. So here, of course, the velocity of combination is determined by the product $k_1 \cdot I_t \cdot E_f$ as well as by $k_2$ $(EI)$. But introducing $i$ as a measure of the *fractional* inhibition has the effect of removing $E_f$ and $(EI)$, while the requirement that the same value of $i$ be attained at equilibrium in all cases fixes the concentration $I_t$ in such a way that the product $k_1 \cdot I_t$ always bears the same proportionality to $k_2$. Thus for a series of inhibitors with the same $k_2$, while the product $k_1 \cdot I_t$ always remains the same, $k_1$ and $I_t$ individually may vary reciprocally without influencing the forward reaction velocity. On the other hand, if $k_2$ changes, the product $k_1 \cdot I_t$ must also change, resulting in a new forward reaction rate.

The mechanical analogy depicted in *Figure 2* may help explain why a forward reaction rate should depend upon a reverse velocity constant. Water is to be run into an empty tub at a constant rate from a reservoir so large that its water level does not fall when the tub fills. The inflow rate can be varied by changing either the reservoir level or the size of the inflow tap, or both. A steady state is ultimately to be reached, with the tub exactly filled to a certain pre-arranged level (e.g. half full). The inflow tap may not be tampered with after having once been opened to the desired degree. What determines the rate of filling of the tub?

[1] O. H. Straus and A. Goldstein, J. Gen. Physiol. *26*, 559 (1943). – A. Goldstein, J. Gen. Physiol. *27*, 529 (1944).

Obviously, an outlet from the tub is essential, for otherwise the water would ultimately overflow no matter how slow the inflow rate. If a *very small* outlet is provided, the requirements of the problem can be met, but only in one way—by running the water in *very slowly*. Of course, the inflow rate from the start must always be exactly the same as the outflow rate at the steady state; and the latter will depend entirely upon the size of the outlet, if the tub is always to be filled to the same level. If the outlet is enlarged the inflow rate can be increased correspondingly and the tub will fill more quickly. The rate of filling is thus always determined by the outlet size, and since the inflow rate can be adjusted by altering either or both of two factors (reservoir level, inflow tap), neither of these *alone* has any bearing on the result.

Now let us suppose that the tub is supplied with outlet and inflow taps that are *both* fixed, so that only the reservoir level can be varied. The analogy is now complete, for both the rate-determining constants are fixed characteristics of the system just as are $k_1$ and $k_2$ for a given enzyme-inhibitor pair. The mechanical problem can still be solved by selecting the right reservoir level, and this maneuver corresponds exactly with varying $I_t$ to discover the concentration needed to produce a desired degree of inhibition at equilibrium. As before, the outlet size governs the rate of filling by determining the required inflow rate, and one is forced to choose that single reservoir level which will yield this inflow rate. In a series of such mechanical systems, all tubs with the same outlet size will fill (to the same degree) at the same rate, regardless of the size of their inflow taps. All tubs with inflow taps of the same size (or whose reservoir levels are the same) will fill at different and unpredictable rates, unless their outlets happen to be the same. Therefore in all tubs, the rate of filling (to the same degree) will always be predictable from the outlet size alone, and will be slower, the smaller the outlet.

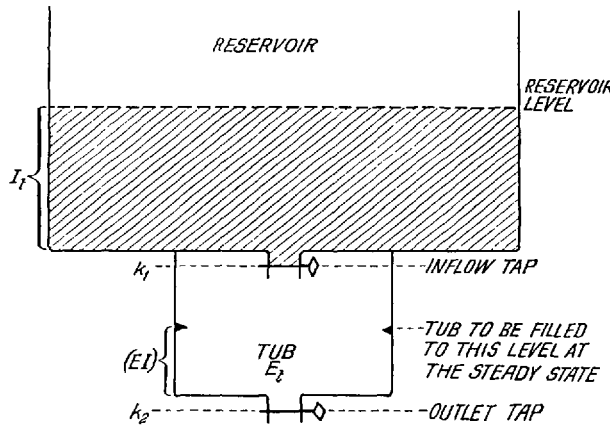


Fig. 2.–*Mechanical analogy to rate of development of inhibition.* For explanation see text.

It is of some interest to consider the physical meaning of $k_2$, upon which the rate of development of inhibition depends. This constant expresses the tendency of the enzyme-inhibitor complex to dissociate. It is therefore necessarily a measure of intrinsic stability of the complex, i.e. of the tightness with which inhibitor molecules are bound to enzyme. In thermodynamic usage it has been customary to define bond strength in terms of the free energy change described by the equilibrium constant ($\Delta F = -RT \ln K$), i.e. to equate *tightness of binding* with *affinity*. It must be noted, however, that while

the equilibrium constant defines the overall tendency to form a complex (the resultant of associating and dissociating tendencies), it gives no information about how tightly or loosely the inhibitor molecules are bound. It is the property measured by $k_2$ that has to do with the strength of the intermolecular forces holding inhibitor molecules at the enzyme surface, and this may vary quite independently of the affinity.

Consider aliquots of an enzyme solution in two vessels containing, respectively, inhibitors $X$ and $Y$. If both inhibitors have the same affinity for the enzyme, and if their concentrations in the two vessels are the same, then the two enzyme solutions will be inhibited to the same degree when equilibrium is reached. But this tells us nothing about the relative tendencies of $EX$ and $EY$ to dissociate. $X$, for example, might combine very readily (e.g. by virtue of a high proportion of effective collisions = large $k_1$) and form a loose complex (= large $k_2$) so that at equilibrium the molecules of $X$ would be associating with and dissociating from the enzyme with great frequency. $Y$, on the other hand, might combine only slowly (e.g. because of steric hindrance = small $k_1$) yet form an extremely tight complex (= small $k_2$) so that at equilibrium the frequency of association and dissociation would be much lower.

It was established earlier that the rate of approach to equilibrium is directly determined by $k_2$, and it has now been pointed out that $k_2$ is a measure of the tightness of the enzyme-inhibitor bond (in the sense defined above). It therefore follows that if a series of inhibitors is under study, those that are bound most tightly to enzyme (i.e. those with smallest values of $k_2$) will not only dissociate *but also associate* most slowly. In general, for the type of system considered here, the tighter the enzyme-inhibitor bond, the more slowly will inhibition develop. Conversely, an unusually slow development of inhibition indicates the formation of a very tight complex[1].

Since this paper was submitted for publication, MYERS[2] has elaborated a theoretical approach to the kinetics of systems where inhibition is brought about by competitive substrates with very low turnover rates. In such cases, which may be more common than formerly supposed, $k_2$ might be negligibly small, the reaction being represented by the equation $E + I \underset{}{\overset{k_1}{\rightarrow}} EI \overset{k_5}{\rightarrow} E +$ products. Despite the different reaction mechanism, the foregoing analysis should remain valid, except that the rate-determining role will now be played by $k_5$ instead of $k_2$. In this situation the rate of attainment of equilibrium will still depend upon the "dissociation" rate of $EI$, which is equivalent to the rate of destruction of inhibitor.

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*Zusammenfassung*

Bei gewissen reversiblen Reaktionen wird, unter bestimmten, stark eingeschränkten, aber doch häufig vorkommenden Bedingungen, die Geschwindigkeit der Gleichgewichtseinstellung für die *Hin*-Reaktion nur durch die Geschwindigkeitskonstante der *Rück*-Reaktion bestimmt. Diese besondere Anwendung des Massenwirkungsgesetzes wird an der Hemmungsentwicklung in gewissen Enzym-Inhibitor-Systemen illustriert.

In einem reversiblen Enzym-Inhibitor-System, in dem der grössere Teil der Inhibitormoleküle frei bleibt, erfolgt die Einstellung eines gegebenen Hemmungsgrades um so langsamer, je fester das Inhibitormolekül im entstehenden Komplex gebunden ist. Kann der Hemmungsgrad gegen die Zeit aufgetragen werden (vom Augenblick der Mischung von Enzym und Inhibitor an gerechnet), so lässt sich aus der erhaltenen Kurve die Geschwindigkeitskonstante der Dissoziation berechnen, selbst wenn die Konzentrationen der Reaktionsteilnehmer unbekannt sind.

[1] It should be emphasized again that these conclusions do not apply to the case where most inhibitor molecules are combined at equilibrium (apparent stoichiometric reversible combination) or to irreversible inhibition.

[2] D. K. MYERS, Biochem. J. (in press, 1952).

## Congressus

### SCHWEDEN

### XIII. Internationaler Kongress für reine und angewandte Chemie in Stockholm, 1953

Die 17. Konferenz der Internationalen Union für reine und angewandte Chemie und der 13. Internationale Kongress der reinen und angewandten Chemie werden im Sommer 1953 vom 29. Juli bis 4. August in Stockholm und vom 5. bis 7. August in Uppsala stattfinden. Der Kongress beschränkt sich auf das Gebiet der physikalischen Chemie mit den Unterabteilungen: Chemische Thermodynamik und Thermochemie, Elektrochemie, Oberflächen- und Kolloidchemie, Reaktionskinetik und andere Gebiete der physikalischen Chemie. Ferner wird ein Symposion über Holzchemie arrangiert mit den Untergruppen: Strukturchemie der Holzbestandteile, Chemie der Zellulose und der Hemizellulose, Ligninchemie. Unmittelbar nach dem Hauptkongress in Stockholm wird in Uppsala ein Symposion der makromolekularen Chemie abgehalten.

## CONSTRUCTIONES

### TSCHECHOSLOWAKEI

### Neues hydrobiologisches Institut in Sedlice

Am 31. Mai 1952 wurde in Sedlice bei Blatná ein hydrobiologisches Institut eröffnet, welches unter der Leitung von Dr. RUDOLF ŠRÁMEK-HUŠEK steht. Assistent ist Dr. Jiří Růžička. Das Institut verfügt über biologische und chemische Laboratorien, eine grosse Bibliothek, Photoräume und Aquarienanlagen. Das Arbeitsprogramm betrifft – entsprechend der Lage des Institutes in ausgedehnten See- und Teichgebieten – regionale Limnologie, insbesondere Vergleiche verschiedener Teichbiocönosen und Studium einiger praktischer Organismengruppen (Infusorien, Cladoceren, Copepoden, Desmidiaceen). Die 1925 von Prof. K. SCHÄFERNA† gegründete Hydrobiologische Station an den Lnáře-Teichen liegt 12 km vom neuen Institut entfernt und steht mit diesem als Feldstation in Verbindung. Die Adresse der neuen Station lautet: Hydrobiologisches Institut der Akademie, Sedlice bei Blatná, Československo.

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