The Effect of the Steady-State on the Kinetic Analysis of Enzyme Inhibitors That Are Not Competitive*

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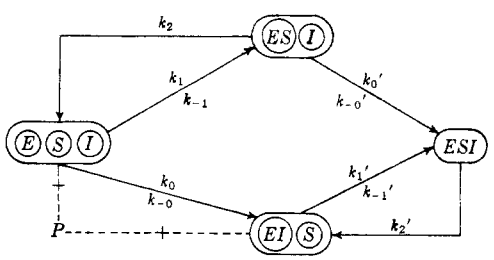
The effects of different degrees of deviation from quasi-equilibrium on the kinetic analysis of a system containing an inhibitor that possesses not competitive character was tested. Increasing deviations from the linear, quasi-equilibrium v versus $v/(S)$ plots were observed. The effect of changing the individual rate constants, especially those associated with the not competitive inhibition, was determined. The amount of deviation was found to increase as the not competitive nature of the concentration of the inhibitor increased. The use of these deviations as a test for quasi-equilibrium was found to provide an indicative test.

It has been pointed out (Botts and Morales, 1953; Morales, 1955) that reciprocal plots of velocity, v, and substrate concentration, (S), are not linear in the presence of inhibitors that possess other than strictly competitive character unless certain conditions are met. In this paper, the type of inhibition that is other than competitive will be referred to as "not competitive" rather than noncompetitive or uncompetitive. The generalized mechanism for inhibition of enzyme catalysis is given in Scheme I. Three simultaneous equations [(1), (2), and (3)] have been deduced (Botts and Morales, 1953) from this mechanism by application of the steady-state treatment to the free enzyme, the enzyme-substrate complex, the enzyme-inhibitor complex, and the enzyme-substrate-inhibitor complex:



Scheme I

$$k_{-0}W_{EI} + (k_{-1} + k_2)W_{ES} = 0 \quad (1)$$

$$(k'_{-1} + k'_2)W_{ESI} - [(k'_{-1} + k'_2)\bar{K}'_I(S) + k_{-0}]W_{EI} = (r - r')k'_{-1}K'_0\bar{K}_1(I)(S) \quad (2)$$

$$k'_{-0}W_{ESI} - [k'_{-0}K'_0(I) + (k_{-1} + k_2)]W_{ES} = 0 \quad (3)$$

where $W_{EI} = \frac{(EI)}{(E)} - K_0(I)$, $W_{ES} = \frac{(ES)}{(E)} - \bar{K}_1(S)$, and $W_{ESI} = \frac{(ESI)}{(E)} - K'_0\bar{K}_1(I)(S)\bar{K}_1 = \frac{k_1}{k_{-1} + k_2}$, $\bar{K}'_1 = \frac{k'_1}{k'_{-1} + k'_2}$, $K_0 = \frac{k_0}{k_{-0}}$, $K'_0 = \frac{k'_0}{k'_{-0}}$, $r = \frac{k_2}{k_{-1}}$ and $r' = \frac{k'_2}{k'_{-1}}$.

If for any reason equation (2) is equal to zero, equations (1), (2), and (3) are homogeneous; this means that generally all the W's are zero. Equation (2) can be zero for a number of reasons, some trivial such as $(S) = 0$, and some not. In any case where equation (2) is zero, the velocity will be given by equation (4). If equation (2)

$$v = \frac{[k_2\bar{K}_1(S) + k'_2K'_0\bar{K}_1(S)(I)](E_0)}{1 + K_0(I) + \bar{K}_1(S) + \bar{K}_1K'_0(S)(I)} \quad (4)$$

is zero because (I) is zero, equation (4) reduces to the familiar Briggs-Haldane expression for v. If equation (2) is zero because of steric blockage of S by I or of I by S—*i.e.*, $K'_0 = K'_1 = 0$—equation (4) becomes the equation of competitive inhibition. Thus, the quasi-equilibrium assumption does not have to be made in order to obtain an expression which can be put in a linear reciprocal form if the inhibition is strictly competitive. On the other hand, it must be assumed that the binding of S is unaffected by bound I and the binding of I is unaffected by bound S—*i.e.*, $K_0 = K'_0$—and also that both r and r' approach zero before equation (2) is zero when an inhibitor which is not competitive is present. The resulting rate expression can be put in the linear reciprocal form only if these assumptions—the quasi-equilibrium assumptions—are made.

If equation (2) does not equal zero, the resulting expression for v is given by equation (5).

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$$v = \frac{[k_2\bar{K}_1(S) + k'_2K'_0\bar{K}_1(S)(I)](E_0) + \dfrac{k_{-0}k'_{-1}(r - r')[k_2k'_{-0} + k'_2(k'_0(I) + k_{-1}(1 + r))]K'_0\bar{K}_1(S)(I)}{k_{-0}k'_{-1}(1 + r')[k'_0(I) + k_{-1}(1 + r)] + k'_{-0}k_{-1}(1 + r)(k'_1(S) + k_{-0})}(E_0)}{1 + K_0(I) + \bar{K}_1(S) + \bar{K}_1K'_0(S)(I) + \dfrac{k'_{-1}(r - r')[(k_{-0} - k'_{-0})k_{-1}(1 + r) + k_{-0}k'_{-0}(1 + K'_0(I))]K'_0\bar{K}_1(S)(I)}{k_{-0}k'_{-0}(1 + r')[k'_0(I) + k_{-1}(1 + r)] + k'_{-0}k_{-1}(1 + r)(k'_1(S) + k_{-0})}} \quad (5)$$

It is apparent then that in the steady-state neither reciprocal plots of v and (S) nor the more useful v versus $v/(S)$ plots will be linear if an inhibitor possessing not competitive character is present unless certain conditions are met (Botts and Morales, 1953). The velocities calculated from the equation based on the quasi-equilibrium assumption [equation (4)] will be different from those calculated from equation (5). The quantitative differences of these velocities calculated from the two assumptions at different degrees of deviation from the quasi-equilibrium assumption, different relative values of the competitive and not competitive nature of the inhibition, and different relative values of the individual rate constants, holding their ratios, the equilibrium constants, constant, will be determined. The effects of varying concentrations of not competitive inhibitor will be determined. The values obtained with equation (4) will be plotted in the v versus $v/(S)$ form and the straight lines compared to similar plots obtained from equation (5). The possibility of using the deviations observed when the quasi-equilibrium is not operative as a test for quasi-equilibrium will be examined.

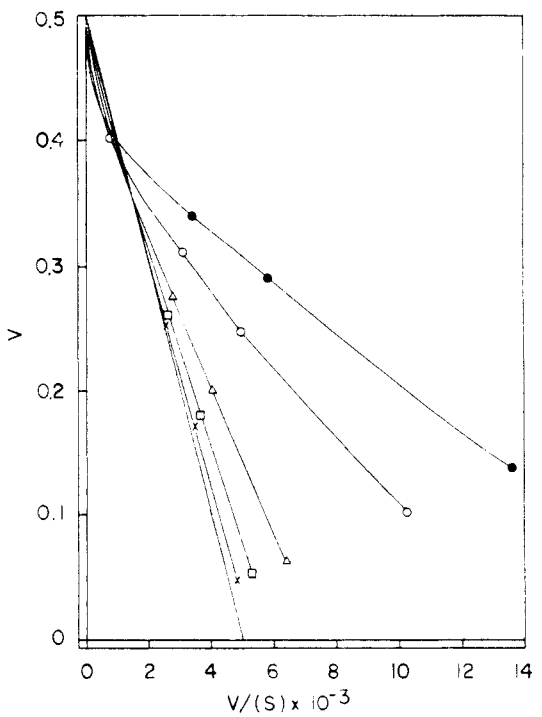

FIG. 1.—Velocity is plotted versus velocity divided by the substrate concentration for an inhibited system when the following parameters are constant: $\bar{K}_1 = 1.0 \times 10^4$/M, $k_2 = 1.0$, $K'_1 = 1.0 \times 10^4$/M, $k'_1 = 10^4$, $k'_{-1} = 1.0$, $k'_2 = 0.0$, $r' = 0.0$, $(I)K_0 = 1.0$, $k_0 = 2 \times 10^4$, $k_{-0} = 2.0$, $(I)K'_0 = 1.0$, $k'_0 = 1.0 \times 10^4$, $k'_{-0} = 1.0$.

The following table lists the parameters which are changing in the plots and the apparent values of V'_m and K'_m which were calculated from the apparently linear portions of the plots:

Line	$k_1 \times 10^{-4}$	k_{-1}	r	K'_m (apparent) $\times 10^4$ M	V'_m (apparent)
———	$\gg 1$	$\gg 1$	$\rightarrow 0.0$	1.00	0.50
×—×	3.0	2.0	0.5	0.92	0.49
□—□	2.0	1.0	1.0	0.73	0.47
△—△	1.5	0.5	2.0	0.56	0.44
○—○	1.2	0.2	5.0	0.29	0.40
●—●	1.1	0.1	10.0	0.20	0.41

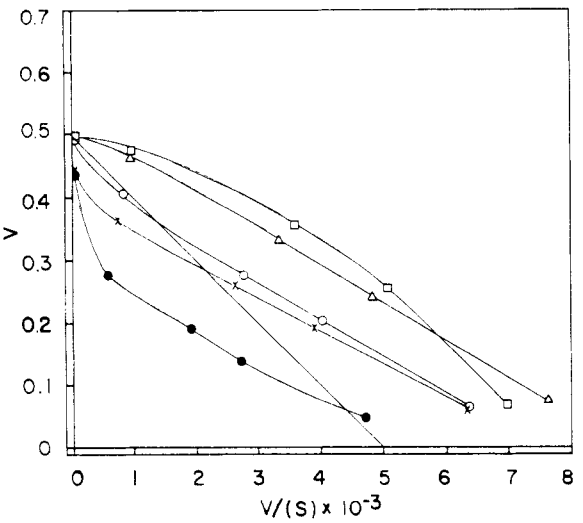

FIG. 2—Velocity is plotted versus velocity divided by the substrate concentration for an inhibited system when the following parameters are constant: $\bar{K}_1 = 1.0 \times 10^4$/M, $k_1 = 1.5 \times 10^4$, $k_{-1} = 0.5$, $k_2 = 1.0$, $r = 2.0$, $\bar{K}'_1 = 1.0 \times 10^4$/M, $k'_1 = 1.0 \times 10^4$, $k'_{-1} = 1.0$, $k'_2 = 0.0$, $r' = 0.0$, $(I)K_0 = 1.0$, $(I)K'_0 = 1.0$.

The following table lists the parameters which are changing in the plots and the apparent values of V'_m and K'_m which were calculated from the apparently linear portions of the plots:

Line	$k_0 \times 10^{-4}$	k_{-0}	$k'_0 \times 10^{-4}$	k'_{-0}	K'_m (apparent) $\times 10^4$ M	V'_m (apparent)
○—○	2.0	2.0	1.0	1.0	0.56	0.44
×—×	20.0	20.0	1.0	1.0	0.53	0.40
□—□	0.20	0.20	1.0	1.0	—	—
△—△	2.0	2.0	10.0	10.0	0.59	0.52
●—●	2.0	2.0	0.10	0.10	0.57	0.30

Solid line is the quasi-equilibrium plot.

RESULTS

Figure 1 is a v versus $v/(S)$ plot for several values of r (k_2/k_{-1}). When $r \rightarrow$ zero (the quasi-equilibrium assumption) the plot is linear. When r is increased to 0.5, small deviations from the quasi-equilibrium line occur. As r is increased from

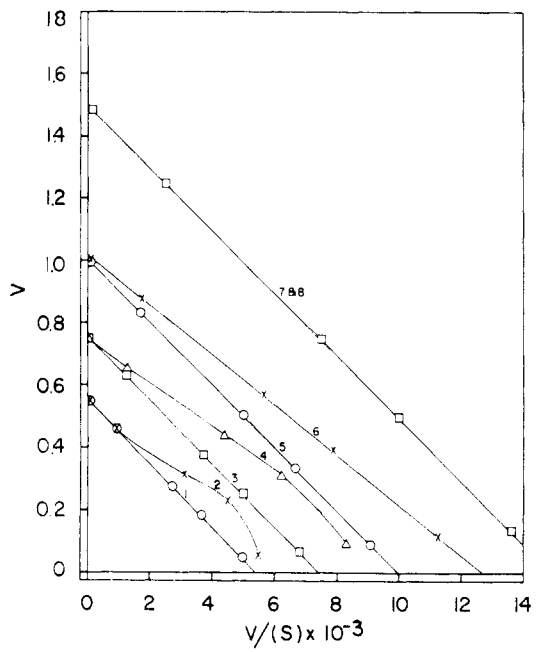

FIG. 3.—Velocity is plotted versus velocity divided by substrate concentration for an inhibited system when the following parameters are constant: $\bar{K}_1 = 1.0 \times 10^4$/M, $k_2 = 1.0$, $(I)K_0 = 1.0$, $k_0 = 2.0 \times 10^4$, $k_{-0} = 2.0$, $(I)K'_0 = 1.0$, $k'_0 = 1.0 \times 10^4$, $k'_{-0} = 1.0$.

The following table lists the parameters which are changing in the plots and the apparent values of K'_m and V'_m which were calculated from the apparently linear portions of the plots.

Line No.	$k_1 \times 10^{-4}$	k_{-1}	r	$\bar{K}'_1 \times 10^{-4}$ M	$k'_1 \times 10^{-4}$	k'_{-1}	k'_2	r'	K'_m (apparent) $\times 10^4$ M	V'_m (apparent)
1	$\gg 1$	$\gg 1$	$\rightarrow 0.0$	1.0	1.0	$\gg 0.1$	0.1	0	1.00	0.55
2	1.5	0.5	2.0		1.0	1.0	0.1	0.1	—	—
3		$\gg 1$	0.0		1.0	$\gg 0.5$	0.5	0	1.00	0.75
4	1.5	0.5	2.0		1.0	1.0	0.5	0.5	—	—
5		$\gg 1$	0.0		1.0	$\gg 1$	1.0	0	1.00	1.00
6	1.5	0.5	2.0		1.0	1.0	1.0	1.0	0.79	1.00
7		$\gg 1$	$\rightarrow 0.0$		1.0	$\gg 2.0$	2.0	0	1.00	1.50
8	1.5	0.5	2.0		1.0	1.0	2.0	2.0	1.00	1.50

0.5 to 10.0, increasing deviations from the quasi-equilibrium line are observed. When (S)'s within an order of magnitude of the actual K'_m in the presence of the inhibitor are used, the deviations are very pronounced, whereas (S)'s two orders of magnitude greater than K'_m show less deviation. Since velocity data are usually taken in the (S) range where (S) approximates K'_m, significant deviation in the observed and subsequently plotted velocity will occur if r is greater than 0.5. Figure 1 illustrates, however, that at any value of r tested, the v versus $v/(S)$ plot will appear to be linear unless very wide ranges of (S) are used. The slope of these apparently straight lines will not equal $-K'_m$, and the extrapolated V'_m will not be the true limiting velocity at infinite (S). The legend under Figure 1 lists the approximated values of these parameters taken directly from the apparently straight lines determined by the points representing the lower three or four (S)'s at values of r between zero and 10.0. The V'_m is

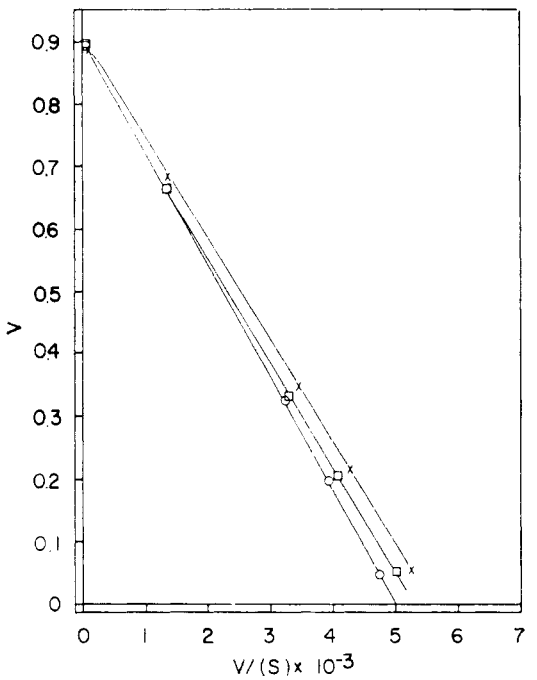

FIG. 4.—Velocity is plotted versus velocity divided by substrate concentration for an inhibited system when the following parameters are constant: $\bar{K}_1 = 1.0 \times 10^4$/M, $k_2 = 1.0$, $\bar{K}'_1 = 1.0 \times 10^4$/M, $k'_1 = 1.0 \times 10^4$, k'_{-1}, $k'_2 = 0.0$, $r' = 0.0$, $(I)K_0 = 1.0$, $k_0 = 2.0 \times 10^4$, $k_{-0} = 2.0$.

The following table lists the parameters which are changing in the plots:

Line	$k_1 \times 10^{-4}$	k_{-1}	r'	$(I) \times K'_0$	$k'_0 \times 10^{-4}$	k'_{-0}
O—O		$\gg 1$	$\rightarrow 0$	0.1	1.0	10.0
×—×	1.5	0.5	2.0	0.1	1.0	10.0
□—□	1.5	0.5	2.0	0.1	1.10	1.0

consistently less than its actual value; at high values of r it is reduced 20%. The K'_m is also consistently less than its actual value; at high r it is reduced as much as five-fold.

Figure 2 is a v versus $v/(S)$ plot when $r = 2.0$ and $K_0 = K'_0$. The individual rate constants, k_0, k_{-0}, k'_0 and k'_{-0} are changed tenfold, but their ratios, k_0/k_{-0} and k'_0/k'_{-0}, are held constant. In each case, increased deviations from the quasi-equilibrium line result when the order of magnitude of the unprimed rate constants is different from that of the primed constants. When the unprimed constants are greater than the primed constants, the curves are concave up, but the deviation in v is not always negative. When the primed constants are greater, the curves are concave down and the observed velocity is greater than that which would be observed if quasi-equilibrium were operative. The legend under Figure 2 lists the approximated values of the extrapolated maximum velocity and the slope of the plots $(-K'_m)$ which would be observed from the points that appear to define a straight line. Again the extrapolated maximum velocity deviates from the actual V'_m, this time by as much as 40% even when r is only 2.0. The estimated

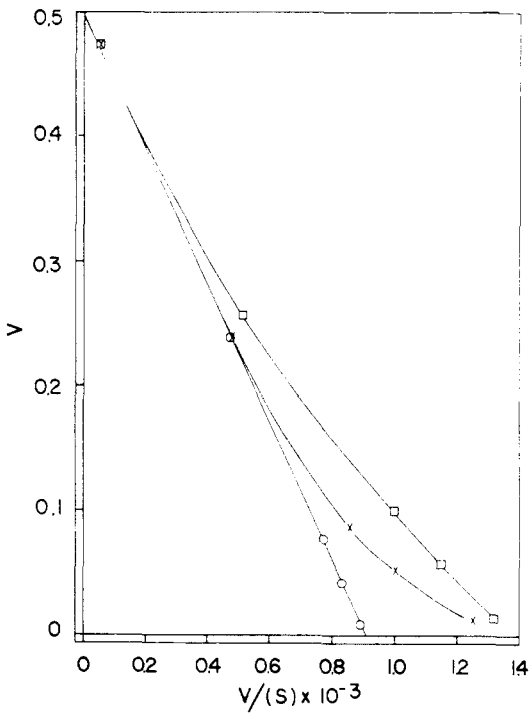

FIG. 5.—Velccity is plotted versus velocity divided by substrate concentration for an inhibited system when the following parameters are constant: $\bar{K}_1 = 1.0 \times 10^4$/M, $k_2 = 1.0$, $\bar{K}'_1 = 1.0 \times 10^4$/M, $k'_1 = 1.0 \times 10^4$, $k'_{-1} = 1.0$, $k'_2 = 0.0$, $r' = 0.0$, $(I) \times k'_0 = 1.0$, $k'_0 = 1.0 \times 10^4$, $k'_{-0} = 1.0$.

The following table lists the parameters which are changing in the plots.

Line	$k_1 \times 10^{-4}$	k_{-1}	r	$(I) \times K_0$	$k_0 \times 10^{-4}$	k_{-0}
O—O		$\gg 1$	$\to 0$	10.0	2.0	0.20
×—×	1.5	0.5	2.0	10.0	2.0	0.20
□—□	1.5	0.5	2.0	10.0	20.0	2.0

K'_m from these plots is about half the actual K'_m at this value of r.

Figure 3 is a v versus $v/(S)$ plot for the steady-state for different values of r'/r when $r = 2.0$. The corresponding plots for quasi-equilibrium are given for different ratios of k'_2/k_2 when $k_2 = 1.0$. In the special case where $r = r'$, no deviation is observed. This represents a coincidental situation that makes equation (2) equal to zero. In the other examples presented, r' is less than r, and the velocities are greater than those expected at quasi-equilibrium. The extrapolated maximum velocity may be very nearly equal to the actual V'_m, especially when r is close to r'. When r is different from r', it is difficult to imagine the resulting plots as being linear. The legend under Figure 3 lists the approximated values of K'_m and V'_m estimated from the plots that appear to define a linear relationship. The K'_m estimated in this manner is again much less than the actual value.

Figures 4 and 5 are v versus $v/(S)$ plots for $r = 2.0$ and $K'_0 = 0.1\ K_0$. In these plots, the competitive nature of the inhibition is ten times greater

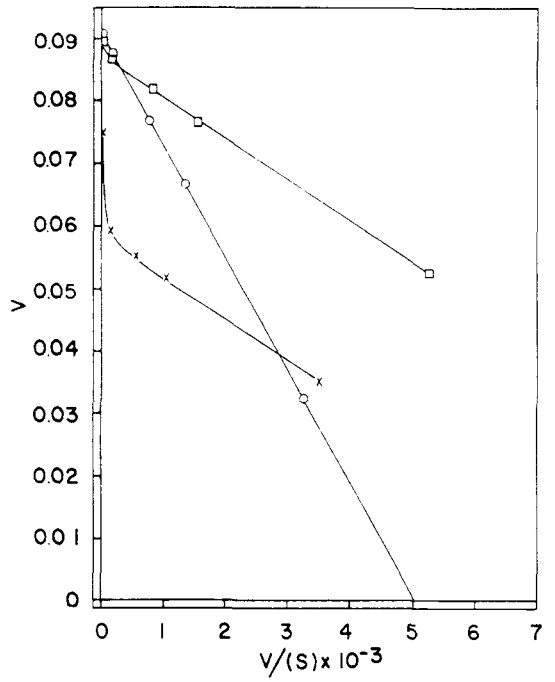

FIG. 6.—Velocity is plotted versus velocity divided by substrate concentration for an inhibited system when the following parameters are constant: $\bar{K}_1 = 1.0 \times 10^4$/M, $k_2 = 1.0$, $\bar{K}'_1 = 1.0 \times 10^4$ M, $k'_1 = 1.0 \times 10^4$, $k'_{-1} = 1.0$, $k'_2 = 0.0$, $r' = 0.0$, $(I)\ K_0 = 1.0$, $k_0 = 1.0 \times 10^4$, $k_{-0} = 1.0$, $(I)K'_0 = 10.0$.

The following table lists the parameters which are changing in the plots and the apparent values of V'_m and K'_m which were calculated from the apparently linear portions of the plots.

Line	$k_1 \times 10^{-4}$	k_{-1}	r	k'	k'_{-0}	K'_m (apparent) $\times 10^4$ M	V'_m (apparent)
O—O		$\gg 1$	$\to 0$	1.0	0.10	1.82	.091
×—×	1.5	0.5	2.0	1.0	0.10	0.64	.087
□—□	1.5	0.5	2.0	10.0	1.0	0.67	.059

than the not competitive nature and consequently should overshadow the not competitive effects. In Figure 4, K'_0 is reduced tenfold because either k'_0 is reduced or k'_{-0} is increased tenfold whereas K_0 has the same value as in the preceding examples. In Figure 5, K_0 is increased tenfold because either k_0 is increased or k_{-0} is decreased tenfold whereas K'_0 has the same value as in Figures 1, 2, and 3. Since no deviations are expected when the inhibitor is strictly competitive, it is expected that these examples would show less deviation from the quasi-equilibrium situation than the preceding ones. As expected, the deviations in Figures 4 and 5 are scarcely discernible, though in Figure 5 they become significant at very low (S).

Figures 6 and 7 are v versus $v/(S)$ plots for $r = 2.0$ and $K'_0 = 10\ K_0$. In these plots, the competitive nature of the inhibition is one tenth the not competitive nature and consequently it should be overshadowed by the not competitive effects. In Figure 6, K'_0 is increased tenfold because either k'_0 is increased or k'_{-0} is decreased

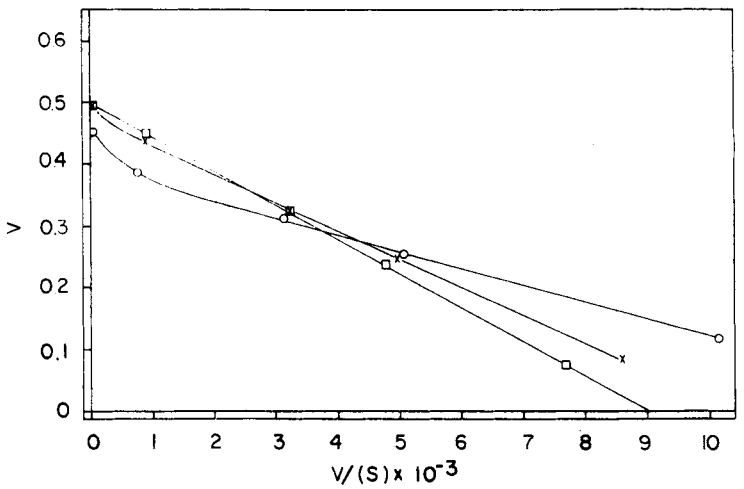

FIG. 7.—Velocity is plotted versus velocity divided by substrate concentration for an inhibited system when the following parameters are constant: $\bar{K}_1 = 1.0 \times 10^4$/M, $k_2 = 1.0$, $\bar{K}'_1 = 1.0 \times 10^4$/M, $k'_1 = 1.0 \times 10^4$, $k'_1 = 1.0$, $k'_2 = 0.0$, $r' = 0.0$, $(I)K_0 = 0.10$, $(I)K'_0 = 1.0 \times 10^4$, $k'_{-0} = 1.0$.

The following table lists the parameters which are changing in the plots and the apparent values of V'_m and K'_m which were calculated from the apparently linear portions of the plots.

Line	$k_1 \times 10^{-4}$	k_{-1}	r	k_0	k_{-0}	K'_m (apparent) $\times 10^4$ M	V'_m (apparent)
□—□		$\gg 1$	$\rightarrow 0$	2.0	20.0	0.56	0.50
○—○	1.5	0.5	2.0	2.0	20.0	0.37	0.40
×—×	1.5	0.5	2.0	0.20	2.0	0.45	0.47

tenfold whereas K_0 has the same value as in Figures 1, 2, 3, and 4. As expected, the deviations in this example are very large. The legend under Figure 6 lists the approximated values of the limiting maximum velocity and the negative of the apparent slope which would be obtained from these plots at the lower substrate concentrations if they were taken to be linear. The deviations observed in Figure 7 are not as large as those in Figure 6, but they are, as expected, quite large. The legend under Figure 7 lists the approximated values of the limiting velocity at infinite (S) and K'_m which would be obtained from these plots at the lower (S) if they were taken as linear.

The type of inhibitor where K_0 does not equal K'_0 has been described (Dixon and Webb, 1958) as the mixed type. If K'_0 is less than K_0 and quasi-equilibrium is operative, the K'_m should increase with increasing (I). If quasi-equilibrium does not exist but K_0 is sufficiently greater than K'_0, the deviations in K'_m should be small. On the other hand, if K'_0 is greater than K_0 and quasi-equilibrium is operative, the K'_m should decrease with increasing (I). In this case, if quasi-equilibrium does not exist, the apparent K'_m observed in a narrow (S) range will be greater than the actual K'_m and will increase somewhat with increasing (I) (see Fig. 8). Thus, the net effect may be that K'_m appears to remain constant or even increase with increasing (I) even though K'_0 is greater than K_0. It is possible then for an inhibitor which is actually of the mixed type where K'_0 is greater than K_0 to appear as a strictly noncompetitive inhibitor or as a mixed type where K'_0 is less than K_0.

Figure 8 illustrates the effect of (I) on the shape of v versus $v/(S)$ plots when $K_0 = K'_0$. Increasing concentrations of inhibitor cause greater deviations so that both the extrapolated limiting velocity and the apparent slope would appear to decrease more than they actually should. Since the slopes $(-K'_m)$ should be constant for strictly noncompetitive inhibition and decrease with inhibitor concentration only in the case of the mixed type of inhibitor (Dixon and Webb, 1958), a purely noncompetitive inhibitor may appear to be of the mixed type if quasi-equilibrium is not operative. The legend under Figure 8 lists the limiting velicities and K'_m's which would be determined from the apparently straight lines at lower (S) from the plots given in Figure 8.

The mechanism upon which equation (5) is based is homeomorphic with several other mechanisms often found in enzyme kinetics. Two enzymes acting on a single substrate or two substrates being acted upon by one enzyme are two such examples. However, these possibilities require that nonlinear v versus $v/(S)$ plots be obtained in the absence of I. Therefore, a nonlinearity induced specifically by the addition of an inhibitor possessing a high degree of not competitive character is an indication that,the system in question does not function at quasi-equilibrium. Correspondingly, if such an inhibitor is added and linearity of v versus $v/(S)$ plots persists, the system

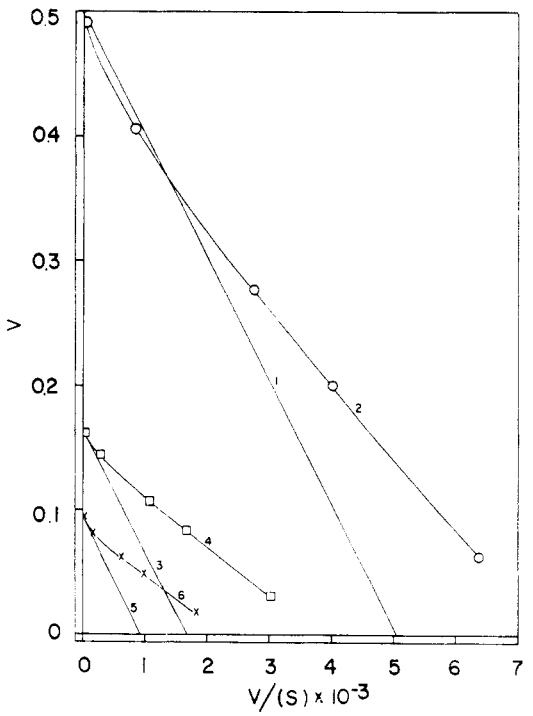

FIG. 8.—Velocity is plotted versus velocity divided by substrate concentration where the following parameters are constant: $\bar{K}_1 = 1.0 \times 10^{-4}$/M, $k_2 = 1.0$, $\bar{K}'_1 = 1.0 \times 10^4$ M, $k'_1 = 1.0 \times 10^4$, $k'_{-1} = 1.0$, $k'_2 = 0.0$, $r' = 0.0$, $k_0 = 2.0 \times 10^4$, $k_{-0} = 2.0$, $k'_0 = 1.0 \times 10^4$, $k'_{-0} = 1.0$.

The following table lists the parameters or concentrations of inhibitor which are changing in the plots and the apparent values of V'_m and K'_m which were calculated from the apparently linear portion of the plots.

Line No.	$k_1 \times 10^{-4}$	k_{-1}	r	$(I) \times 10^4$ M	K'_m (apparent) $\times 10^4$ M	V'_m (apparent)
1		$\gg 1$	$\rightarrow 0$	1.0	1.00	0.50
2	1.5	0.5	2.0	1.0	0.62	0.46
3		$\gg 1$	$\rightarrow 0$	5.0	1.00	0.16
4	1.5	0.5	2.0	5.0	0.51	0.16
5		$\gg 1$	$\rightarrow 0$	10.0	1.00	0.09
6	1.5	0.5	2.0	10.0	0.37	0.09

is functioning close to quasi-equilibrium.

The importance of measuring velocities over a wide range of (S) after the addition of an inhibitor that possesses not competitive character is apparent. If (S)'s at least two orders of magnitude greater than the actual K'_m are not used, the data may lead to meaningless results.

This paper has considered single-substrate kinetics as the first approximation of the results to be expected in more complicated systems. Bi-substrate kinetic analysis in the steady-state reveals that v versus $v/(S)$ plots are expected to be nonlinear even in the absence of inhibitors unless quasi-equilibrium obtains or certain other conditions are met. This point is implied in equation (5) of this paper when I is the second substrate. Usually an ordered sequence of substrate addition to the enzyme is assumed in order to circumvent this difficulty.

Both the Michaelis-Menten equation and the equation presented by Botts and Morales (1953) deal with single-substrate kinetics if an inhibitor is present. Mathematical difficulties persist unless simplifying assumptions such as the quasi-equilibrium assumption or the ordered sequence assumption are made, if more than one substrate is present.

Several examples of data that give nonlinear velocity versus velocity divided by substrate concentration plots may be found in the literature (Edelhoch and Coleman, 1955; Kielley and Kielley, 1953; Fromm and Nelson, 1962; Zewe and Fromm, 1962). If the data reported by Edelhoch and Coleman for the inhibition of RNAse by the products of the hydrolysis is plotted in the v versus $v/(S)$ form, the resulting curves deviate more and more from an approximate straight line as the concentration of inhibitor is increased. Similarly, the data of Kielley and Kielley reported for the inhibition of ATPase by ADP deviate more and more from linearity as the ADP concentration is increased. In both these cases the plots are linear in the absence of inhibitor. In the case of ATPase, if the data between 0.2 and 0.4 μM substrate are used to construct the v versus $v/(S)$ plot, an apparently straight line is formed and the intercept appears to be higher than it actually is. Lower substrate concentrations give plots of steeper slope which, if extrapolated to infinite substrate concentration, give even larger apparent limiting velocities. If the data between 0.2 and 0.4 μM substrate are used, the extrapolated maximum velocity is approximately equal to the V_m in the absence of ADP. This observation could lead to the fallacious conclusion that the inhibition is competitive. Though higher substrate concentrations were not tested, it would be expected that they would give curves which are less steep and which extrapolate to lower limiting velocities. Only at very high substrate concentrations would the true V'_m be revealed. The data observed by Kielley and Kielley at low substrate concentration confirm the prediction that deviation from the linearity expected at quasi-equilibrium becomes greater as the substrate concentration is decreased. The data with both ATPase and RNAse confirm the prediction that the deviation from linearity should increase as the inhibitor concentration is increased.

Fromm and Nelson (1962) have observed nonlinear reciprocal plots of velocity and substrate concentration when certain inhibitors are added to the ribitol dehydrogenase reaction. In the absence of these inhibitors the reciprocal plots were linear (Nordlie and Fromm, 1959). These workers suggest that an ordered sequence of substrate addition occurs with ribitol dehydrogenase. The appropriate coenzyme binds the enzyme prior to binding by either substrate, ribitol or ributol. The effect of the ordered sequence is to simplify the kinetic equations for the

formation and breakdown of the ternary complex so that reciprocal plots of velocity versus either substrate concentration are linear even if quasi-equilibrium is not obtained provided an inhibitor possessing not competitive character is not present.

Zewe and Fromm (1962) have reported a study of the lactate dehydrogenase reaction. Examination of the reciprocal plots reported by these workers shows that they are slightly nonlinear, even in the absence of added inhibitor. This result is suggested by equation 5 when a two substrate enzyme system adds the substrates randomly or even preferentially provided quasi-equilibrium is not obtained. The data of Zewe and Fromm also show that this non-linearity in the reciprocal plots is greatly exaggerated when certain inhibitory products are added.

Fromm and associates suggest that the reduced or oxidized coenzymes form abortive ternary complexes with the fully reduced or fully oxidized enzyme substrate complexes. These inhibitory products do not form complexes only with the free enzyme then but also interact with the complex between the second substrate and the enzyme. Thus, these inhibitors would be expected to have not-competitive character. This, in turn, would be expected to induce a non-linearity in the reciprocal plots or, if the plots were already non-linear, to aggravate that non-linearity. The data reported by Zewe and Fromm (1962) clearly show that increasing concentrations of these inhibitory products cause increasing curvature in the reciprocal plots. This confirms the prediction that the deviations increase as the inhibitor concentration increases and also as the ratio of inhibitor concentration to inhibitor dissociation constant increases.

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Diffusion Measurements in Agar Gel

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An improved method for the employment of agar gel as a medium for diffusion measurements and a mathematical treatment of the data for the calculation of diffusion coefficients are described. Diffusion is allowed to proceed under controlled conditions into a column of agar gel held in a cell made of a 50-ml hypodermic syringe that has had the needle end of the barrel cut off. By means of a micrometer the gel is pushed from the cell, sectioned with a fine wire, and assayed. The use of arithmetic probability paper affords a convenient means of plotting the data, making necessary correction for the presence of the gel, and calculating diffusion coefficients. Advantages include the possibility of using mixtures and very dilute solutions. Coefficients obtained on some salts, sugars, amino acids, and proteins are in agreement with those obtained by free diffusion.

The employment of gels as media for the study of diffusion under circumstances where concentrations are very low and where mixed solutes are present appears to have good possibilities. The rigidity of the gel enables sharp boundaries to be produced and controls to a great extent the errors resulting from thermal or mechanical mixing. Gels can be sectioned accurately, and precise analyses for several substances can be made on each section. In contrast, other methods employed for studies on mixed solutes, such as diffusion through a porous disk (Northrop and Anson, 1929), or free diffusion with various means of layer analysis (Bourdillon, 1941; Cohen and Bruins, 1924; Polson, 1944), either do not allow a study of concentration patterns or are subject to the usual difficulties in forming boundaries and preventing mixing. The use of gels, however, is not without some complications. The particles that make up the structure occupy space and thereby reduce the solvent volume within unit volume of gel. The particles also get in the path of diffusing solute molecules and thereby increase

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