Molecular Order of Participation of Inhibitors (or Activators) in Biological Systems

Abstract. *From a Hill plot it is frequently possible to determine whether exactly one, two, or several inhibitor (or activator) molecules are involved in a critical rate-determining biological process.*

Fifty years ago Hill (*1*) correctly interpreted the sigmoid curve for oxygen saturation of hemoglobin as resulting from cooperative forces that made a partially saturated hemoglobin molecule unstable with respect to either a fully saturated or fully unsaturated hemoglobin molecule. Subsequently similar evidence for cooperativity was found in denaturation of proteins (*2*). More recently such sigmoidal dependence of enzyme activity on substrate concentration has been observed in a wide variety of enzymes together with sigmoidal dependence on various inhibitors or activators (*3*). Such effects have been generally described as allosteric, and the viewpoint has developed that the enzyme consists of a number of subunits with some degree of conformational interaction, such that the binding of one substrate molecule or inhibitor molecule or activator molecule facilitates the binding of subsequent ligands.

Hill (*1*) analyzed the experimental data for the binding of oxygen to hemoglobin and showed that the data fit the equation

$$\log y = n \log x + K \qquad (1)$$

y being the percentage saturation of hemoglobin with oxygen, x the partial pressure of oxygen, and n (the slope) an experimentally determined number which is a combination of the number of reactive sites and the extent of interaction between them. Thus, although mammalian hemoglobins have four interacting subunits, n is found experimentally to be about 2.9 or 3.0, indicating less than total interaction or, conversely, some stability for the partially saturated forms.

Changeux (*4*), Taketa and Pogell (*5*), and especially Atkinson (*6, 7*) have done much to extend the analysis to enzymic activity and to give more reality to the meaning of n. Atkinson (*7*) has shown how the Hill equation can be modified for only partial inhibition of the enzyme and how nonintegral values of n should be expected in most cases. The coefficient n will be a whole integer only when the enzyme inhibitor complex containing fewer than n (that is, the number equivalent to n) molecules of inhibitor is fully active, or when the cooperative binding of successive inhibitor molecules is so great as to yield essentially no enzyme inhibitor complexes containing fewer than n inhibitor molecules.

We would like to add several observations on the application of the Hill equation to enzymic or even cellular reactions. If the usual algebraic operations are applied to reactions that are being inhibited by competitive, noncompetitive, and uncompetitive inhibitors, one derives three equations respectively:

$$\log\left(\frac{1}{V} - \frac{1}{V_0}\right) = n \log [\mathrm{I}] + \log \frac{K_m}{V_{max}\,[\mathrm{S}]\,K_I} \qquad (2)$$

$$\log\left(\frac{1}{V} - \frac{1}{V_0}\right) = n \log [\mathrm{I}] + \log \frac{K_m + [\mathrm{S}]}{V_{max}\,K_I\,[\mathrm{S}]} \qquad (3)$$

$$\log\left(\frac{1}{V} - \frac{1}{V_0}\right) = n \log [\mathrm{I}] + \log \frac{1}{K_I\,V_{max}} \qquad (4)$$

where V is the inhibited rate, V_0 is the uninhibited rate, n (the slope) is the interaction coefficient, [I] is the concentration of inhibitor, [S] is the concentration of substrate, K_m is the Michaelis constant, V_{max} is the uninhibited rate at maximum substrate concentrations, and K_I is the dissociation constant for the enzyme inhibitor complex. In actual practice it is generally simplest to determine the ratio of uninhibited rate to inhibited rate (V_0/V) for a given set of conditions. It is immediately apparent that

$$\frac{V_0}{V} - 1 = \frac{V_0}{V} - \frac{V_0}{V_0} = V_0\left(\frac{1}{V} - \frac{1}{V_0}\right) \qquad (5)$$

Log $V_0 + \log [(1/V) - (1/V_0)]$ is the experimentally accessible expression of inhibition and is plotted on the ordinate of Figs. 1–4. To modify the left side of Eqs. 2, 3, and 4 in order to use the function derived in Eq. 5 it is only necessary to add

$$\log V_0 = \log \frac{V_{max}\,[\mathrm{S}]}{K_m + [\mathrm{S}]}$$

to the left and right sides respectively. Thus

$$\log V_0 + \log\left(\frac{1}{V} - \frac{1}{V_0}\right) = n \log [\mathrm{I}] + \log \frac{K_m}{K_I\,(K_m + [\mathrm{S}])} \qquad (6)$$

$$\log V_0 + \log\left(\frac{1}{V} - \frac{1}{V_0}\right) = n \log [\mathrm{I}] + \log \frac{1}{K_I} \qquad (7)$$

$$\log V_0 + \log\left(\frac{1}{V} - \frac{1}{V_0}\right) = n \log [\mathrm{I}] + \log \frac{[\mathrm{S}]}{K_I\,(K_m + [\mathrm{S}])} \qquad (8)$$

In each case 50 percent inhibition is found at that value of [I] where the left-hand side of the equation is equal to zero and in each case the plot is linear in log [I] with a slope of n. However, there are distinctions between competitive (Eq. 6), noncompetitive (Eq. 7) and uncompetitive (Eq. 8) inhibition according to how the inhibition is affected by changing substrate concentrations. Thus, if the inhibition is noncompetitive, the value of the left-hand side of the equation is independent of substrate concentration. If the inhibition is competitive, the value of the left-hand side will be proportional to

$$\log\left(\frac{K_m}{K_I\,(K_m + [\mathrm{S}])}\right)$$

or, at higher concentrations of substrate S, proportional to $-\log [\mathrm{S}]$. Thus, although inhibition (or activation) involving several molecules of inhibitor (or activator) is not amenable to graphical analysis by the Lineweaver and Burk (*8*) or Eadie (*9*) methods, the logarithmic or Hill-type plot establishes which of the major kinds of inhibition is involved. A mathematical proof of the superiority of the Eadie plot as a means of deriving enzymic constants has been presented by Dowd and Riggs (*10*).

In most recent discussions, it has been implied that these higher order effects of inhibitors or activators involve multiples of subunits in which conformational changes are propagated

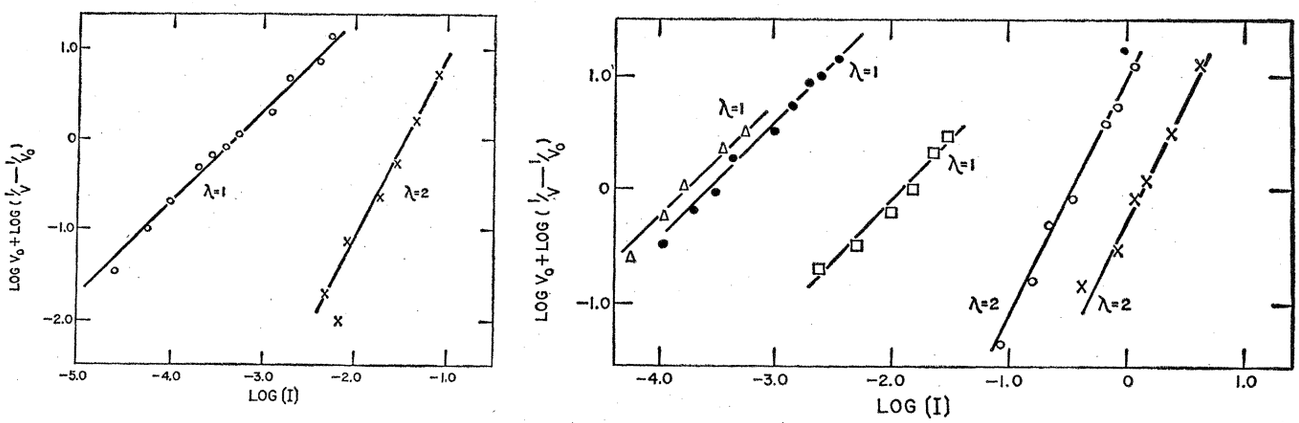

Fig. 1 (left). The inhibition of alcohol dehydrogenase (yeast) by diethyldithiocarbamate (×) or *o*-phenanthroline (○). The data are recalculated from studies by Vallee and coworkers (*11*). The slopes are constant and exactly 1.0 or 2.0 over a broad range of reaction rate and inhibitor concentration, an indication that exactly one phenanthroline molecule or exactly two diethyldithiocarbamate molecules probably are required to inactivate one enzymic center. Fig. 2 (right). The inhibition of *E. coli* valine-activating enzyme by several nucleophilic inhibitors. The reactions studied include: inhibition of ATP-inorganic pyrophosphate exchange by hydroxylamine (×), by imidazole (○) and by *o*-phenanthroline (□); inhibition of valyl-tRNA synthesis by pyrophosphate ion (△); and inhibition of valine hydroxamate synthesis by pyrophosphate ion (●) (*12*). In each case the plotted data fall exactly on straight lines of slope 1.0 or 2.0 indicating that exactly one or exactly two molecules of inhibitor inactivate one enzymic site.

from one subunit to another. There is nothing in the derivation of the equations that precludes the action of several inhibitor or activator molecules in a cooperative manner at the single active site of the enzyme. Figures 1 and 2 show two examples of situations where either exactly 1.0 or exactly 2.0 molecules of inhibitor are reacting with one molecule of enzyme. Although alcohol dehydrogenase (Fig. 1) from liver probably contains two subunits (*11*), it would appear that the subunits do not interact, because exactly 1.0 molecule of phenanthroline is required to inactivate one active site of the enzyme. The fact that the diethyldithiocarbamate slope is exactly 2.0 is doubly significant. If it takes two molecules of diethyldithiocarbamate to achieve what is done by one molecule of *o*-phenanthroline, it seems likely that this potentially tridentate molecule, $(C_2H_5)_2$=NCSSH, is functioning only as a monofunctional nucleophilic agent. Beyond this, the fact that the slope is exactly 2.0 casts doubts on the possi-

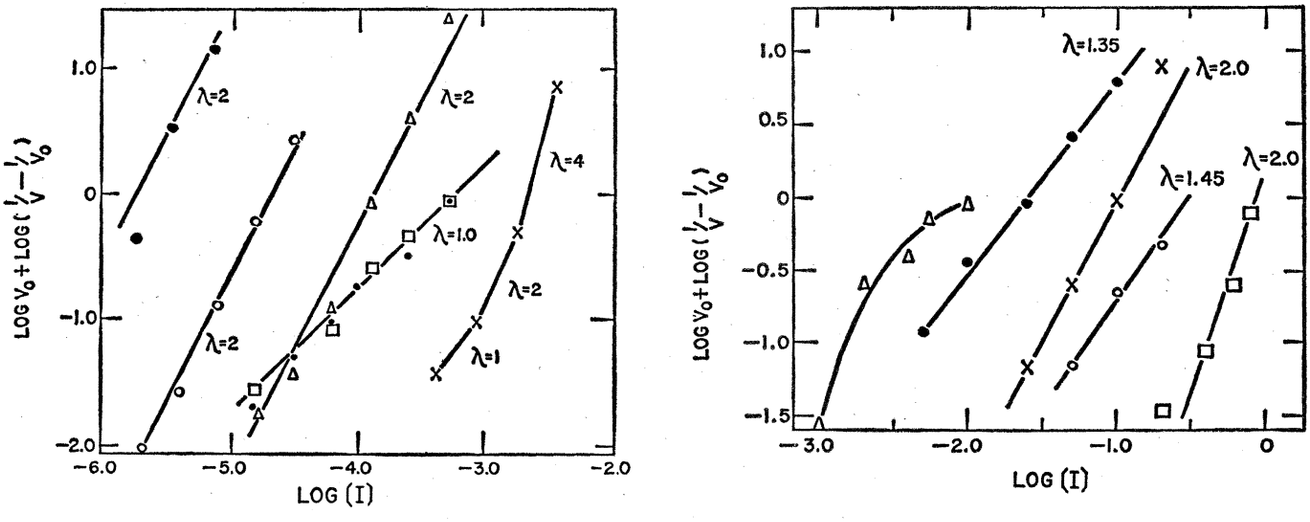

Fig. 3 (left). The inhibition of *Arbacia punctulatus* cell division by several phenols and by iodoacetate. Several of the published experiments of Clowes and co-workers (*14*) have been replotted according to the Hill-type equation. Amino phenol (•) and dinitrocarvacrol (□), fall on the same line with a slope of 1.0. Triiodophenol (●), tribromophenol (○), and dibromophenol (△) fall on quite different straight lines each with a slope of 2.0. We interpret this as strong suggestive evidence that either one or two molecules, respectively, of phenol inactivate a single function critical to cell division. Iodoacetate (×) yields a curve of changing slope, indicating that several vital functions are inactivated, depending on inhibitor concentration. Fig. 4 (right). The inhibition of brain tissue respiration by pentanol (●), butanol (×), propanol (○), ethanol (□), and acetaldehyde (△), as determined by Beer and Quastel (*15*). Two of the alcohols yield straight lines of slope 2, suggesting that some critical center reacts cooperatively with two molecules of butanol or ethanol to inhibit respiration. The other two alcohols yield slopes greater than one but less than two, suggesting that cooperativity is less than complete for pentanol and propanol. However, the constancy of slope suggests that only a single site is involved in the concentration range studied. Acetaldehyde yields an entirely different curve whose changing slope suggests that an acetaldehyde-enzyme complex may have substantial residual activity or that other modes of oxidation are available at higher concentrations of acetaldehyde and similar compounds.

bility of interactions between subunits. It is believed that there are only two subunits in alcohol dehydrogenase from liver. A slope of 2.0 could be obtained only if the interaction were total. Since there is no sign of subunit interaction in the case of *o*-phenanthroline, it seems more likely that both molecules of diethyldithiocarbamate are involved in attacking a single site on the enzyme and that one or the other of the Atkinson (*7*) conditions obtains; either a subunit with one molecule of diethyldithiocarbamate is fully active or the association constant for the second molecule of inhibitor is so great as to make the population of enzyme bound to one molecule of inhibitor negligible compared to that of enzyme or enzyme bound to two molecules of inhibitor.

Figure 1 is concerned with an enzyme for which there is excellent evidence of metal participation (Zn^{++}), as well as independent evidence that one *o*-phenanthroline molecule inactivates one site. Figure 2 shows the properties of a less well-studied enzyme, valine transfer RNA ligase (*12*). Here again, there is a striking linear relation between log $[(1/V) - (1/V_0)]$ and log [I]. In each case the slope (λ) has an integral value of 1.0 or 2.0, an indication that it requires two molecules of hydroxylamine or imidazole to achieve what one molecule of *o*-phenanthroline or one pyrophosphate ion can do. Notably, much higher concentrations of the monofunctional bases are required to inhibit enzyme function. Again the integral values of the slopes may be interpreted as total subunit interaction, but it seems more likely that a single independent active site (containing metal?) is reacting with one bidentate or two monofunctional molecules of inhibitor. [This inference is supported by the lack of any reports of subunit structures among amino acid activating enzymes (*13*)]. In general, a precise adherence to a slope of integral value over an extremely broad range may be suggestive evidence of multiple attack on a single site, rather than cooperative attack on conformationally related sites since such cooperativity is unlikely to be complete.

Much more complicated biological systems may be amenable to similar study. If any number of inhibitor molecules interferes with any single step in a vital process, the Hill plot should have a constant slope whose value may or may not be integral according to the conditions mentioned by Atkinson above. On the other hand, if several steps are affected by the inhibitor (with different association constants), it would be expected that the slope of the Hill plot would increase with increasing concentrations of inhibitor as more vital centers are affected.

In Fig. 3, we have plotted the recalculated data of Clowes and his colleagues (*14*) on inhibition of *Arbacia* cell division. The mechanism of action of the phenolic inhibitors is not well established, but probably involves formation of adenosine triphosphate (ATP) rather than having a direct effect on one of the mitotic events. Nonetheless, each of the six phenols plotted in Fig. 3 exhibits linearity over a 60-fold range of inhibitor concentrations, under conditions where the inhibition varies from 0 to 97 percent.

There are wide variations in the affinity of the inhibitor for the sensitive site (the value on the abscissa corresponding to an ordinate value of zero yields the concentration of inhibitor required for 50 percent inhibition). Nonetheless, each of the phenols appears to react at a single site with exactly one or two molecules of phenol required for total inactivation of whatever process (presumably ATP synthesis) is rate limiting in cell division.

A quite different inhibitor, iodoacetic acid, is included in Fig. 3 for comparison. The Hill plot shows a continuously changing slope, beginning at 1.0 at low concentrations of iodoacetate and increasing to approximately 4.0 at high concentrations. We infer that at low concentrations one iodoacetate ion reacts with a single critical sulfhydryl group and that at higher concentrations, iodoacetate progressively attacks other vital residues of lower reactivity, such that the inhibition is cumulative and appears to be of high order.

We have examined data for a number of inhibitions of complex reactions. In some cases either the data are inadequate or the process is so complex that there is no suggestion of a linear relation. On the other hand, a large number of inhibitory reactions of cellular processes do appear to follow these kinetics. Figure 4 shows how the respiration of brain slices is affected by a number of alcohols and by acetaldehyde (*15*). In this case, each alcohol yields a linear plot with a slope of 1.3 to 2.0 depending on the alcohol. Again it would seem likely that a single site has been attacked and that two molecules of alcohol are involved. In the case of pentanol and propanol, the slope is less than 2.0—from which we infer that the enzyme with only one molecule of alcohol is at least partially active or that the binding of a second molecule of alcohol is not overwhelmingly favored. The curve showing inhibition by acetaldehyde is included for comparison. This curve is dramatically different —either the critical site bound to acetaldehyde is partially active or new oxidative pathways are opened by the acetaldehyde. In any event, it seems likely that the action of acetaldehyde is different from and more complex than the action of aliphatic alcohols.

In summary, first, each of the better characterized types of enzyme inhibition has been shown mathematically to be amenable to a Hill-type treatment in which the logarithm of a function of the degree of inhibition bears a linear relationship to the logarithm of the concentration of inhibitor. Second, in several instances, the slope of such a line has an integral value indicating that exactly one or exactly two (or more) inhibitor molecules react cooperatively to totally inactivate the enzyme. In other cases, the slope does not have an integral value, and this is most easily visualized as being a consequence of incomplete allosteric interaction or incomplete inhibition. Third, in a wide variety of instances (*Arbacia* growth, antibiotic action, alkanol inhibition of brain respiration, and others), Hill plots show linearity over wide ranges of inhibitor concentration. Where such linearity is found, the interpretation is that a single vital process is rate determining and sensitive to a determinable number of inhibitors or activator molecules.

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Solar Radiation Profiles in Openings in Canopies of Aspen and Oak

Abstract. *Vertical profiles of solar radiation in openings in forest canopies showed increasing solar radiation with depth in Colorado, but not in Minnesota. A model was developed and tested to calculate solar radiation in openings from the incoming direct and sky radiation and from the depth and diameter of the opening. The increase occurs only with high direct and low sky radiation. The model may explain the geographic and seasonal distribution of the solar radiation profiles and the lack of such observations previously.*

Vertical profiles of solar radiation in vegetation canopies usually show decreasing intensity with increasing depth into the canopy. The decreasing intensity is largely a function of the decreasing probability of a continuous gap in the foliage to transmit the direct solar beam (*1*, *2*). However, under certain conditions solar radiation was found to increase before decreasing with depth in the canopy, a previously unrecognized anomaly. A mathematical model is presented that accounts for the observations. This model appears to be generally applicable and important primarily in arid regions or at high altitudes.

In June and July 1966, near solar noon I measured vertical profiles of solar radiation in sunlit areas of four small aspen *Populus tremuloides* and two Gambel's oak *Quercus gambelii* stands in west central Colorado at about 2440 m, using the same radiometer at each level in the canopy. The number of measurements which could be made was small. In the summer of 1967, I measured the solar radiation profiles with different solar radiometers, one mounted above and four mounted at different levels within the canopies of one oak and one aspen stand in west central Colorado and in one aspen stand in northern Minnesota. These measurements were made 15 to 20 times during 20 minutes of each daylight hour for periods of 1 to 5 days in each stand in late June and early July and in August. The radiometers were not moved during the measurements in a stand, so they were sometimes shaded during the 20-minute interval. Solar radiation profiles were constructed for each hour from the means of the 15 to 20 measurements at each level. In 1968, measurements were made to test the model proposed here. One radiometer was held manually in gaps in the canopy and repositioned at different levels. Two additional radiometers were permanently mounted outside the canopy, one to measure total downward solar radiation and the other to measure diffuse sky radiation. Signals from both the canopy radiometer and the stationary radiometers were recorded on a multipoint recorder within 10 seconds so that changes in the incoming solar radiation would be recorded. All radiometers were calibrated against an Eppley pyrheliometer.

In 1966, with clear skies, solar radiation in gaps near the top of the canopy increased by 0.05 to 0.10 cal cm^{-2} min^{-1}, or up to 4 to 7 percent greater than the total incoming solar radiation (*3*). The same radiometer was used at all levels, so unbalanced calibration of the radiometers was not the cause of the measured increase. No increase occurred with overcast skies, indicating that the increase was related to the ratio of direct to diffuse solar radiation. In 1967, even though the measurements were made with no effort to insure that the radiometers were in the sun during the measurements, 37 out of 123 profiles indicated higher levels of radiation at some level within the canopy than were recorded above the canopy. In Minnesota none of the 17 profiles measured showed an increase in solar radiation. In Colorado a greater percentage of profiles showed an increase in early summer than in the August measurements. In Colorado in June and early July, 12 of the 32 profiles measured in aspen and 17 of the 30 oak showed an increase; and in August, 5 of the 28 profiles in aspen and 3 of the 16 measured in oak showed an increase

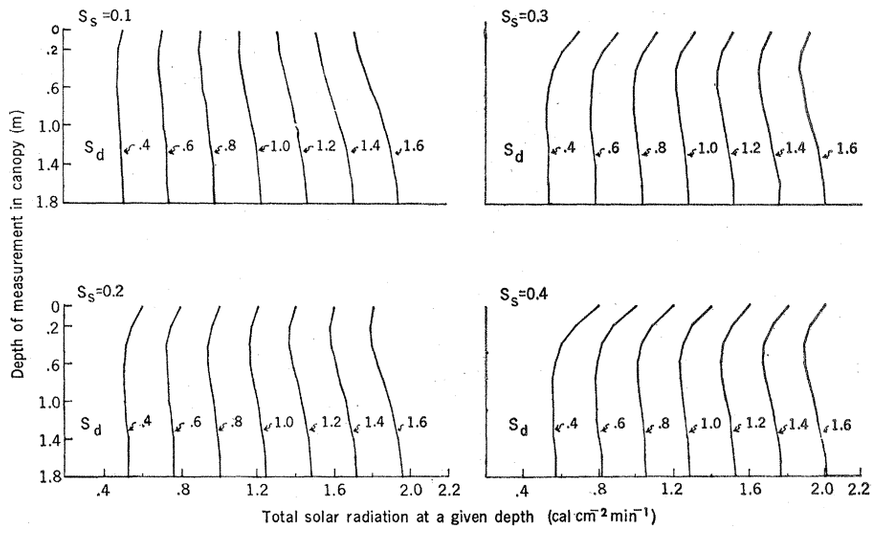

Fig. 1. Total solar radiation at different depths in openings near the top of the canopy calculated for different combinations of six intensities of the incoming solar radiation and four intensities of incoming sky radiation. The curves indicate an increase in total solar radiation in the canopy when the direct beam is high and the sky component is low.