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Ultrasensitivity in Biochemical Systems Controlled by Covalent Modification

INTERPLAY BETWEEN ZERO-ORDER AND MULTISTEP EFFECTS*

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A previous analysis of covalent modification systems (Goldbeter, A., and Koshland, D. E., Jr. (1981) *Proc. Natl. Acad. Sci. U. S. A.* 78, 6840–6844) showed that steep transitions in the amount of modified protein can occur when the converter enzymes are saturated by their protein substrate. This "zero-order ultrasensitivity" can further be amplified when an effector acts at more than one step in a monocyclic or multicyclic cascade of covalent modification. We analyze the limitations of the latter "multistep ultrasensitivity" and show how it can combine with the zero-order effect to enhance the sensitivity of biochemical systems controlled by covalent modification.

In all biological systems, it is necessary to increase or decrease activities in response to external and internal signals. The sensitivity of the system to such signals becomes an important function of the regulatory controls. The terms "ultrasensitivity" and "subsensitivity" (1, 2) have been defined to indicate cases in which the sensitivity is greater or less than that to be expected from the standard hyperbolic (Michaelis-Menten) response.

Three potential sources of ultrasensitivity have been recognized: (*a*) the positive cooperativity of allosteric proteins, (*b*) the multistep effect when the same effector acts at more than one step in a pathway, and (*c*) the zero-order effect in covalent modification when at least one of the converter enzymes is saturated with its protein substrate.

Positive cooperativity was first recognized by Bohr in the case of hemoglobin (3) and is conveniently expressed quantitatively in terms of the Hill coefficient (4). A Hill coefficient as high as 3.8 has been shown in CTP synthetase but most enzymes have appreciably smaller Hill coefficients and many are subsensitive, *i.e.* negatively cooperative (5). The second potential source of ultrasensitivity is exemplified by the effective appearance of cAMP in 5 different steps of the glycogen cascade as revealed in the classic studies of Krebs, Fisher, Cohen and others (6, 7). How much sensitivity is generated by these multiple inputs has not been measured, but it is clearly well below a 5th power dependence on cyclic AMP concentration. Some quantitative aspects of multiple inputs have been developed by Chock and Stadtman (8) and applied to the glutamine synthetase cascade (9). The existence of multiple inputs does not automatically produce ultrasensitivity. The fact that the constants of the effectors must be in a narrow range to produce advantages for multiple-step and allosteric sensitivity has been discussed (1). The third source of ultrasensitivity, *i.e.* the zero-order effect, has been recently uncovered theoretically (2, 10) and demonstrated experimentally in the phosphorylation of isocitrate dehydrogenase (11).

In a highly regulated metabolic system more than one of these effects may be operating at the same time. It is important, therefore, to evaluate the limitations and potentialities of each effect and their interplay. This is particularly important in the case of multistep and zero-order effects since both can (and have been observed in the isocitrate dehydrogenase case to) occur together. In this paper, we shall explore the potentialities and limitations of zero-order and multistep effects and examine how their interplay enhances the sensitivity of a pathway to regulatory control.

THEORY OF ZERO-ORDER AND MULTISTEP EFFECTS

Properties of Zero-order Ultrasensitivity in Covalent Modification

In a covalent modification system in which (Equation 1) a protein W is reversibly modified between W and W^* by converter enzymes E_1 and E_2, the steady-state fraction of modified protein, W^*/W_T, is given by Equation 2. For convenience we shall simply use the mole fraction W^* to mean the ratio (W^*) to W_T. (See Refs. 2 and 10 for a detailed derivation of Equations 2–6, 10, and 12 below).

$$W \underset{E_2}{\overset{E_1}{\rightleftarrows}} W^* \tag{1}$$

$$\left(\frac{W^*}{W_T}\right) = W^* = \frac{\phi + \left[\phi^2 + 4K_2\left(\frac{V_1}{V_2} - 1\right)\left(\frac{V_1}{V_2}\right)\right]^{1/2}}{2\left(\frac{V_1}{V_2} - 1\right)} \tag{2}$$

with

$$\phi = \left(\frac{V_1}{V_2} - 1\right) - K_2\left(\frac{K_1}{K_2} + \frac{V_1}{V_2}\right) \tag{3}$$

Here, $V_1 = k_1E_{1T}$ and $V_2 = k_2E_{2T}$ are the maximum rates of enzymes E_1 and E_2 at a given effector concentration (see "Properties of Multistep Ultrasensitivity in Covalent Modification"); $K_1 = K_{m1}/W_T$ and $K_2 = K_{m2}/W_T$ are the Michaelis constants of E_1 and E_2, divided by the total amount of target protein.

Thus, when V_1/V_2 is increased from an initial low value, the fraction of modified protein progressively rises from a

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value much smaller than unity to a value close to unity when V_1 exceeds V_2. The ratio of modification and demodification rates corresponding to a given value of W^* is given by Equation 4. In particular, the midtransition point, where $W^* = W = 0.5$, corresponds to the ratio of modification rates shown in Equation 5.

$$\frac{V_1}{V_2} = \frac{W^*(1 - W^* + K_1)}{(1 - W^*)(W^* + K_2)} \tag{4}$$

$$\left(\frac{V_1}{V_2}\right)_{0.5} = \frac{1 + 2K_1}{1 + 2K_2} = \frac{W_T + 2K_{m1}}{W_T + 2K_{m2}} \tag{5}$$

The latter equation indicates how the ratio of V_1/V_2 yielding half-maximum modification will be affected by changes in K_{m1} and K_{m2} with respect to W_T. We have previously shown that the transition $W \rightarrow W^*$ becomes steeper and steeper as K_{m1} and K_{m2} decrease below W_T. This phenomenon was termed "zero-order ultrasensitivity" (1, 2) to indicate that the enhancement in sensitivity with respect to a Michaelian response depends on at least one of the converter enzymes operating outside the first-order range.

The effect of altering the relative values of the Michaelis constants K_{m1} and K_{m2} in such a way that one is above and the other below W_T is shown in Fig. 1. It can be seen that the top of the modification curve is steeper when $K_{m1} \ll K_{m2}$, whereas the converse is true when $K_{m1} \gg K_{m2}$. Then the completion of the $W \rightarrow W^*$ transition is very shallow as V_1/V_2 increases, whereas the initial part of the curve is steeper. In addition, as expected from Equation 5, the midpoint of the transition curve is shifted to higher values of V_1/V_2 when K_{m1} increases with respect to K_{m2}.

It is important to evaluate the time dependence of the shift in the amount of modified protein, since regulation must be achieved in physiologically reasonable times. The time evolution of the modification system subsequent to a change in the ratio of V_1/V_2 is governed by the differential Equation 6.

$$\frac{dW^*}{dt} = \frac{V_2}{W_T}\left[\frac{(V_1/V_2)(1 - W^*)}{(K_1 + 1 - W^*)} - \frac{W^*}{K_2 + W^*}\right] \tag{6}$$

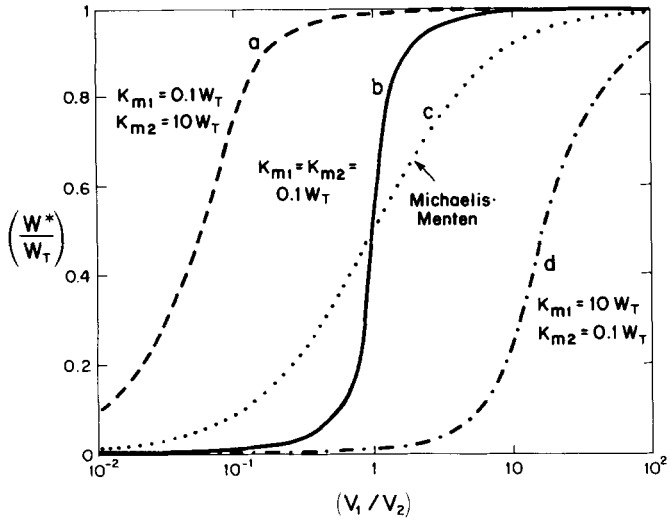

FIG. 1. **Effect of varying ratios of K_{m1} and K_{m2} on zero-order ultrasensitivity in covalent modification.** When the Michaelis constants K_{m1} and K_{m2} are both smaller than W_T, the modification *curve b* is much steeper than the Michaelian-like *curve c*. When one of the modifying enzymes operates in the zero-order domain whereas the other operates in the first-order domain, the curves are still steeper than the Michaelis one, but they become shallower at the bottom (*a*) or at the top (*d*) depending on whether K_{m2} or K_{m1} is greater than W_T.

In Fig. 2 is shown the variation of the fraction of modified protein (W^*/W_T) following instantaneous increases in the ratio (V_1/V_2) when the modifying enzymes operate in the zero-order and the first-order kinetic domains. The increase in the ratio of modification rates is achieved here by an increase in V_1, for a fixed value of V_2. A comparison of parts *a* and *b* shows that in the former case a minor change in the ratio of modification rates (from 0.9 to 1.1) suffices to shift the target protein from 90% unmodified to 90% modified form, whereas a change by a factor of at least 50 is needed in the absence of zero-order effect. When the variation in V_1/V_2 is not particularly rapid, *e.g.* when V_1/V_2 rises to the final value with a half-time of the order of minutes, zero-order ultrasensitivity is associated with the existence of a time lag of similar duration. This time lag in the rise of modified protein (see Fig. 5 of Ref. 2) is due to the time required for V_1/V_2 to pass over the modification threshold, illustrated by Fig. 1 and Fig. 2*a*.

To alter V_1 or V_2, it is necessary to activate or inhibit enzymes E_1 and E_2. To exemplify the effect of the mode of control of these enzymes on the modification system, we shall consider both competitive and noncompetitive inhibition of E_2 by an effector J. The reaction rate in these conditions is respectively expressed in Equations 7 and 8.

$$V_2 = \frac{V_{M2}W^*}{K_2\left(1 + \frac{J}{K_{J2}}\right) + W^*} \tag{7}$$

$$V_2 = \frac{V_{M2}}{\left(1 + \frac{J}{K_{J2}}\right)}\left(\frac{W^*}{K_2 + W^*}\right) \tag{8}$$

In these equations, V_{M2} and K_2 represent the maximum rate and the Michaelis constant of E_2 in the absence of effector; J represents the concentration of this effector; K_{J2} is the inhibition constant. The effect of J is to modify the maximum rate but not the Michaelis constant in noncompetitive inhibition, whereas the reverse obtains for competitive inhibition. Such a difference results in markedly different behavior in modification kinetics, as shown in Fig. 3. The modification curve appears to be steeper in the case of noncompetitive inhibition. Such an effect originates in part from the fact that in competitive inhibition, zero-order ultrasensitivity progressively diminishes as the kinetics of E_2 goes from zero-order into first-order (Equation 7).

Properties of Multistep Ultrasensitivity in Covalent Modification

Monocyclic System—To examine the multistep effect in the absence of complication of zero-order effects, we shall examine the influence of an effector J when the enzymes E_1 and E_2 operate in their first-order region. It has previously been shown that zero-order ultrasensitivity is absent in this range (2).

A simple illustrative case occurs when J is an absolute activator of E_1 and a noncompetitive inhibitor of E_2. In that case the ratio of maximum rates V_1/V_2 is given by Equation 9

$$\frac{V_1}{V_2} = \left(\frac{V_{M1}}{V_{M2}}\right)\frac{J(K_{J2} + J)}{K_{J2}(K_{J1} + J)} \tag{9}$$

where V_{M1} and V_{M2} are the maximum activities of E_1 and E_2 in the presence and absence of J, respectively, and K_{J1} and K_{J2} are the activation and inhibition constants.

The variation of W^* as a function of V_1/V_2 in the first-order region is of Michaelian form, as shown by Equation 10

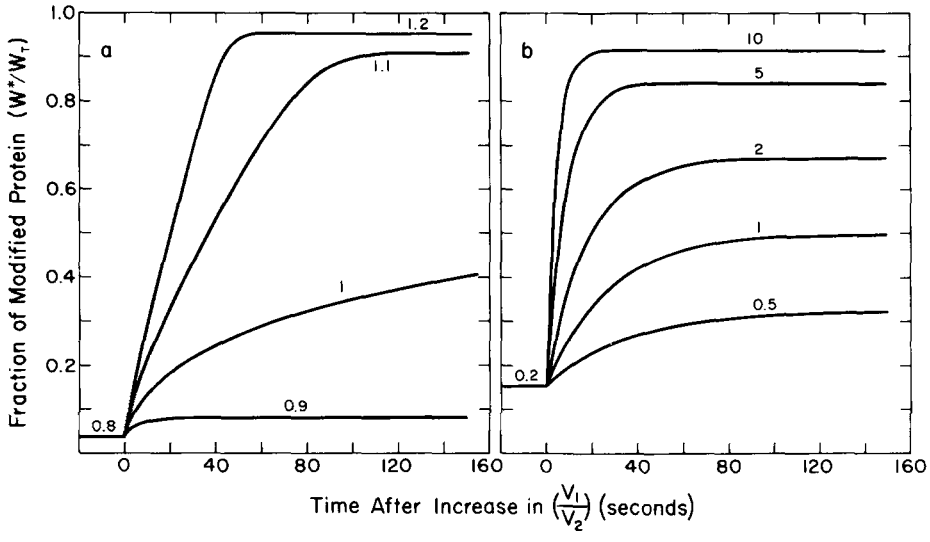

FIG. 2. **Time evolution to steady state of the fraction of modified protein in the domains of zero-order (*a*) and first-order (*b*) kinetics.** The ratios of modification rates V_1/V_2 are changed at time zero by increasing V_1, for example, by addition of an allosteric effector. The initial and final values of V_1/V_2 are shown on the curves for the intervals before and after addition of the effector. The curves are obtained by numerical integration of Equation 6 for $W_T = 100\ \mu M$; *a*, $K_{m1} = K_{m2} = 10^{-2}\ W_T$, $V_2 = 10\ \mu M\ s^{-1}$; *b*, $K_{m1} = K_{m2} = 10\ W_T$ and $V_2 = 20\ \mu M\ s^{-1}$. For an enzyme concentration $E_{2T} = 1\ \mu M$, these values of V_2 correspond to turnover numbers of 10 or 20 s^{-1}, which compare to those observed experimentally. For example, the protein phosphatase I (1 μM) acts on phosphorylase *a* (100 μM) with a $k_{cat} = 3\ s^{-1}$ (7). Phosphorylase kinase (4 μM) acts on glycogen phosphorylase (100 μM) with a $k_{cat} = 158\ s^{-1}$ (7).

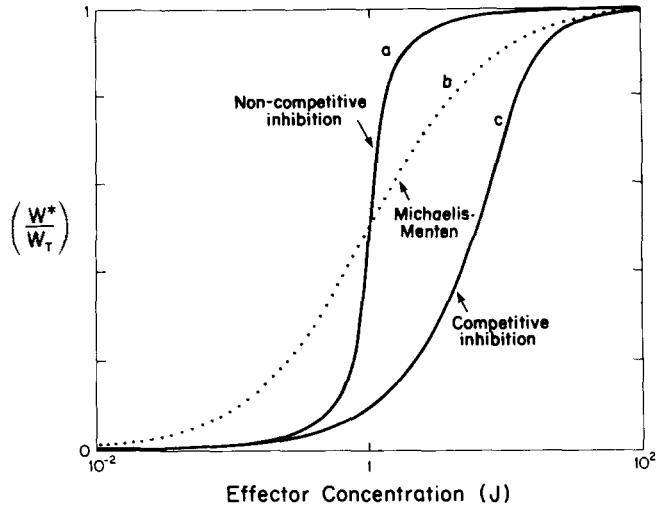

FIG. 3. **Effect of noncompetitive and competitive inhibition of E_2 on the modification curve in the monocyclic system of Equation 1.** The modifying enzymes operate initially in the zero-order domain, $K_{m1} = K_{m2} = 0.1\ W_T$. The variation in the fraction of modified protein is determined as a function of the effector concentration according to Equations 2 and 8 for *a*, with $(V_1/V_{M2}) = 0.1$, $K_{J2} = 0.1\ \mu M$, and according to Equations 2 and 7 for *c*, with $K_{J2} = 0.1\ \mu M$.

(see Ref. 1).

$$\left(\frac{W^*}{W_T}\right) = \frac{V_1/V_2}{\dfrac{K_1}{K_2} + \dfrac{V_1}{V_2}} \tag{10}$$

Taking into account Equations 9 and 10, one can compute the value of the response coefficient R_J (Equation 11) corresponding to the modification curve of W^* as a function of J.

$$R_J = \frac{9\{(9\theta - 1) + [(9\theta - 1)^2 + 36\theta(K_{J1}/K_{J2})]^{1/2}\}}{\{(\theta - 9) + [(\theta - 9)^2 + 36\theta(K_{J1}/K_{J2})]^{1/2}\}} \tag{11}$$

where $\theta = (K_{m1}/K_{m2})/(V_{M1}/V_{M2})$.

The response coefficient R_J (1, 2, 12) has been previously defined as the value of controlling factor, J, which produces 90% of the maximum response in proportion to one that gives a 10% response, *i.e.* $J_{0.9}/J_{0.1}$, where J can be an allosteric effector, a substrate, or a stimulus, etc. The smaller the R_J, the more sensitive is the response. Here, the response coefficient R_J given by Equation 11 corresponds to the 90 and 10% values of the interval from an initial value W^*_i to a final value W^*_f, where the initial and final values are the levels of the modified protein when J goes from zero to infinity. The reason for this, as will be seen below, is that some of the modification curves reach a plateau while the target protein is not fully converted into W^*. A similar flattening of saturation curves has been observed for allosteric interactions (12, 13).

A reciprocal measure of R_J has some advantages, since it increases numerically with increased sensitivity. For a Michaelian system, $R_J = 81$ (1, 2, 12). We have therefore plotted the reciprocal value $81/R_J$ which we call the "relative sensitivity coefficient," as a function of $(K_{m1}/K_{m2})/(V_{M1}/V_{M2})$ in Fig. 4, for different values of K_{J1} and K_{J2}. As described in the figure legend, the relative sensitivity of this index reaches a maximum value of 9, *i.e.* the system is nine times more sensitive than a Michaelian system, when the effector acts in 2 steps, which is the theoretical maximum for a cooperative protein in which the Hill coefficient is 2.

Therefore, it is seen that the optimum multistep effect with J acting in two steps almost reaches the value of 9, which is logical since, from Equation 9, the optimum relationship between V_1/V_2 and the effector involves at most a proportion to J^2. This occurs when K_{J1}/K_{J2} is very large and $(K_{m1}/K_{m2})/(V_{M1}/V_{M2}) \approx 1$. There is actually quite a large plateau region when K_{J1}/K_{J2} is 10^4, which decreases as this ratio decreases. Thus, the lower the ratio of K_{J1}/K_{J2}, the lower the sensitivity. Moreover, it is the ratio of $(K_{m1}/K_{m2})/(V_{M1}/V_{M2})$, not the absolute value of any individual parameter, which controls the sensitivity. The symmetry of the curves is logical since

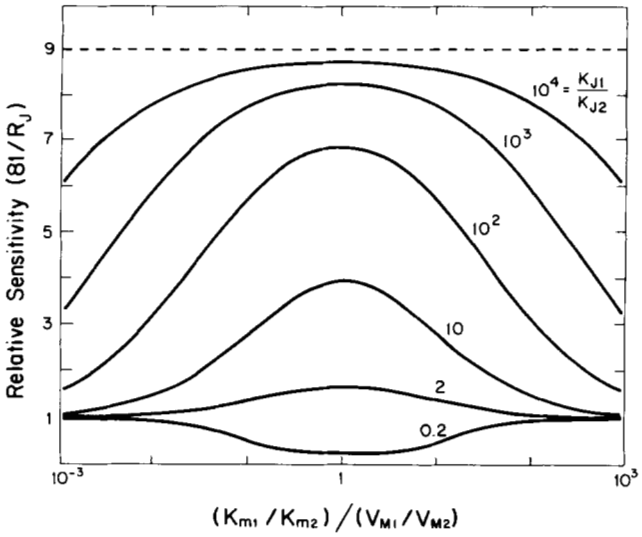

FIG. 4. **Multistep ultrasensitivity in the monocyclic modification system of Equation 1 when effector J activates E_1 and inhibits E_2.** The curves yield the relative sensitivity ($81/R_J$) as a function of the ratio $(K_{m1}/K_{m2})/(V_{M1}/V_{M2})$, for increasing values of the ratio of activation and inhibition constants K_{J1} and K_{J2}. The value of R_J is obtained according to Equation 11. The *dashed horizontal line* on the *top* indicates the limit of the relative sensitivity of an allosteric protein characterized by a Hill coefficient of 2. For an allosteric protein obeying the Hill equation with a Hill coefficient n_H, R_J is given by the formula $R_J = (81)^{1/n_H}$. Hence, the relative sensitivity of such a protein is $81/R_J = 9$.

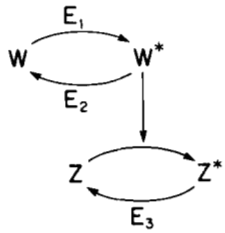

FIG. 5. **Bicyclic modification cascade.** The product of the first modification cycle, W^*, catalyzes the modification of protein Z into Z^* in the second cycle. It is assumed that enzymes E_2 and/or E_3 are inhibited by an effector J but other permutations can be considered.

we are expressing a change from 10 to 90% in steady state, and whether we shift from $W^*/W = 1/9$ to $W^*/W = 9$ or vice versa should be reciprocally related. The curve is invariant when $K_{J1} = K_{J2}$; then indeed, $R_J = 81$ since V_1/V_2 is always proportional to J in that case (as shown in Equation 9), and the sensitivity of the system becomes equivalent to that of a Michaelian process (see Equation 10). The system becomes subsensitive owing to the multistep effect when K_{J1} drops below K_{J2}.

Multistep Effect in a Bicyclic Cascade—It is next desirable to consider a multistep effect in a bicyclic cascade of the type shown in Fig. 5. The protein modified in the first cycle (W^*) catalyzes the $Z \rightarrow Z^*$ conversion in the second cycle. The reversal of that conversion is catalyzed by E_3. To determine the multistep effect alone we assume that all the enzymes operate in the first-order region. J is assumed to be a noncompetitive inhibitor of E_2 and E_3. (An analogous situation of E_2 and E_3 inhibition occurs in glycogen metabolism where the phosphorylated inhibitor protein inhibits the phosphatase which acts on various phosphorylated proteins (14).)

In the specific case shown in Fig. 6, the inhibition constants are assumed to be equal, *i.e.* $K_{J2} = K_{J3} = 0.2$. Other parameters

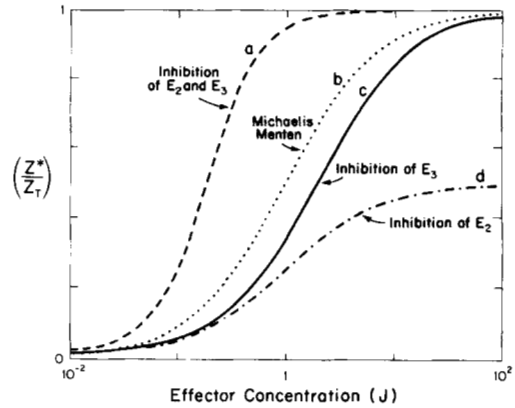

FIG. 6. **Multistep ultrasensitivity in the bicyclic modification cascade of Fig. 5.** *Curves a, c,* and *d* are obtained assuming that J is a noncompetitive inhibitor (see Equation 8) of enzymes E_2 and E_3, of E_3 only, or of E_3 only. Parameter values are $(K_{m1}/K_{m2}) = 10$, $(K_{mW^*}/K_{m3}) = 1$, $(V_{M1}/V_{M2}) = 0.1$, $(V_{MW^*}/V_{M3}) = 1$, and $K_{J2} = K_{J3} = 2.10^{-8}$ M. The curves are obtained in the absence of zero-order ultrasensitivity by successively applying Equation 10 to the first cycle, and a similar equation for Z^* for the second cycle assuming that the ratio (V_{W^*}/V_3) is proportional to the fraction of (W^*/W_T), with $V_{W^*} = V_{MW^*}(W^*/W_T)$.

were selected to give a sizeable multistep effect. *Curves c* and *d* show the effect of inhibition of E_2 alone or E_3 alone. *Curve b* is the Michaelian reference curve. The combination of two inhibitions is shown in *curve a* and gives a response coefficient R_J of 11.6 (or a relative sensitivity of 7). This closely corresponds to the relative sensitivity of a fully cooperative dimeric enzyme.

If J acts only in the first cycle of the bicyclic cascade but on two enzymes (activating E_1 and inhibiting E_2) the change in Z^* is smaller per change in J than the change in W^*. The second cycle in that case diminishes the sensitivity of the first cycle.

It is also seen that inhibition of E_3 is more effective than inhibition of E_2 in relation to the sensitivity of changes in Z^* per change in J. This can be explained qualitatively by the following reasoning. If E_3 is fully inhibited, all Z goes to Z^* as the ratio of modification rates goes to infinity. In contrast, if E_2 is fully inhibited, all W goes to W^*, but this does not cause necessarily all Z to go to Z^*, since E_3 is active. Hence, a controlled cycle followed by a cycle which has no effector control will be less sensitive than a single cycle or a bicyclic cascade in which the last cycle is regulated.

Interplay between the Zero-order and Multistep Effects

Monocyclic System—When an effector acts on two converter enzymes in a system of the type shown in Equation 1, ultrasensitivity could result from a multistep effect, a zero-order effect, or a combination of the two. In such a system the fraction of the substrate protein in the modified form W^* is given by Equation 2 and it is possible to factor out the relative contributions of the multistep and zero-order effects by considering two situations. When K_1 and K_2 are both much larger than unity, *i.e.* when both enzymes are operating in the first order region, it has been established that there is no zero-order effect (2). Thus, a calculation using a low value of (W_T) which satisfies a first-order situation should be able to separate the multistep effect from the zero-order effect. This is shown for an illustrative case in Fig. 7, where *curves b* and *c* show the zero-order and multistep effects alone, and *curve a* shows the combination of the two effects.

To evaluate the zero-order effect alone we have used the

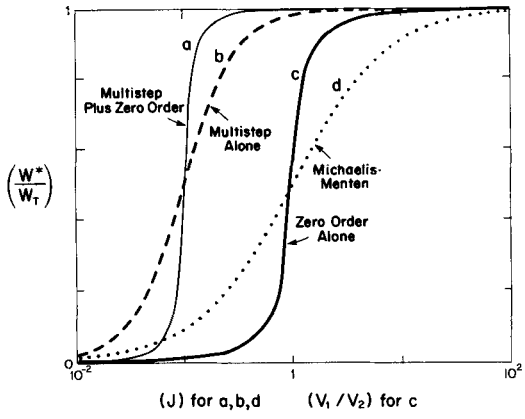

FIG. 7. **Interplay between zero-order and multistep ultrasensitivity in the monocyclic system of Equation 1.** *Curve b* illustrates the multistep effect alone, with $(V_{M1}/V_{M2}) = 1$, $(K_{m1}/K_{m2}) = 1$, $K_{J1} = 1\ \mu M$, and $(K_{J1}/K_{J2}) = 100$. These conditions favor multistep ultrasensitivity (see Fig. 4). The curve is obtained according to Equations 9 and 10. *Curve c* shows the zero-order effect alone. The curve is identical to *curve b* in Fig. 1. *Curve a* results from the combination of multistep and zero-order effects. It is obtained from Equations 2 and 9 for the same parameter values as for *curve b*, with $K_{m1} = K_{m2} = 0.1\ W_T$.

TABLE I

Cumulative effect of multistep and zero-order ultrasensitivity in single covalent modification cycle

The data are obtained for $(K_{m1}/K_{m2}) = (V_{M1}/V_{M2}) = 1.$ and $K_{J1} = 100\ K_{J2} = 1$.

Reduced Michaelis constants of E_1 and E_2	Multistep effect alone		Zero-order ultra-sensitivity alone		Multistep and zero-order cumulated	
$K_1\ (=K_{m1}/W_T) = K_2$	R_J	$\frac{81}{R_J}$	R_V	$\frac{81}{R_V}$	R_J	$\frac{81}{R_J}$
0.01	11.7	6.9	1.2	68.4	1.1	73.8
0.1	11.7	6.9	3.2	25	1.9	42.4
1	11.7	6.9	27.1	3	6.3	12.9
10	11.7	6.9	69.5	1.2	10.7	7.5
100	11.7	6.9	79.7	1.02	11.6	7

Equation 12 obtained in the derivation of the zero-order effect (2).

$$R_V = \frac{81(K_{M1} + 0.1\ W_T)(K_{m2} + 0.1\ W_T)}{(K_{m1} + 0.9\ W_T)(K_{m2} + 0.9\ W_T)} \qquad (12)$$

This equation gives the quantitative relationship for zero-order steepness of the curve regardless of the means of influencing V_1 or V_2. R_V represents the ratio of (V_1/V_2) at 90% of the maximum response *versus* (V_1/V_2) at 10% of the maximum response. The advantage of this formulation is that the effect of changing V_1/V_2 is separated from the method of achieving that change. It is seen from the example of Fig. 7 that most of the ultrasensitivity in that case comes from the zero-order effect but there is a slight additional sensitivity from the multistep effect.

Both effects can be cumulative but they are not simply multiplicative. To evaluate the relative contributions under various circumstances, we have shown in Table I the relative sensitivity indices for the multistep effect alone (column 1), the zero order effect alone (column 2), and the combined effects (column 3) for various values of K_{m1} and K_{m2}.

At large values of K_{m1} and K_{m2} ($K_{m1} = K_{m2} = 100\ W_T$), there is no zero-order effect, and the multistep effect for this particular combination of constants for the cycle yields a R_J value of 11.7. As K_{m1} and K_{m2} decrease, the multistep effect should not change since the only parameter being varied is the concentration of the substrate W_T. This is seen to be true and the zero-order effect becomes more and more significant until a R_V value of 1.2 is reached at $K_{m1} = K_{m2} = 10^{-2}\ W_T$. The R_J value associated with the cumulated zero-order and multistep effects is then close to 1.1. The minimum possible value for R_J is 1, so this is an extraordinarily high ultrasensitivity, corresponding to an allosteric protein with a Hill coefficient of 50. It can be seen from this illustrative example that there is not a simple multiplicative relationship between the two effects but the "global" ultrasensitivity can indeed be greater than either multistep or zero order alone.

How to evaluate the contribution of each effect experimentally becomes apparent from the mathematical relationships described above. One can simply study the kinetics at a value of W_T sufficiently low so that only the multistep effect is observed. Since the parameters of the converter enzymes should not change as a function of the target protein concentration, the relative contribution of multistep ultrasensitivity can be readily ascertained. To determine the contributions of zero-order ultrasensitivity alone, one can calculate the ratio R_V described in Equation 12. The value of R_V can be compared with the overall response as shown in Table I to determine the relative contributions of each effect. This procedure was followed in analyzing the phosphorylation of isocitrate dehydrogenase (11).

The multistep effect will frequently be subsensitive ($R_J >$ 81) but the overall response will be ultrasensitive because of the influence of the zero-order effect. Such a situation is illustrated in Fig. 8 where the transition curve for W^* as a function of the effector J shows an intermediary plateau region. In this case the multistep effect alone is less sensitive than a Michaelis-Menten curve. However, combining such a multistep effect with a zero-order ultrasensitivity equivalent to that of *curve c* in Fig. 7 gives a response close to a Michaelis-Menten curve.

This situation is probably a fairly representative one. Because of the stringent demands on constants for multistep effects, this effect will only rarely be ultrasensitive. On the other hand, activating one step and inhibiting the reverse step is an excellent way to alter V_1/V_2 and thus implement a zero-order effect. The combination of these two phenomena should therefore produce increased sensitivity in many biological systems. As an example we can consider the situation

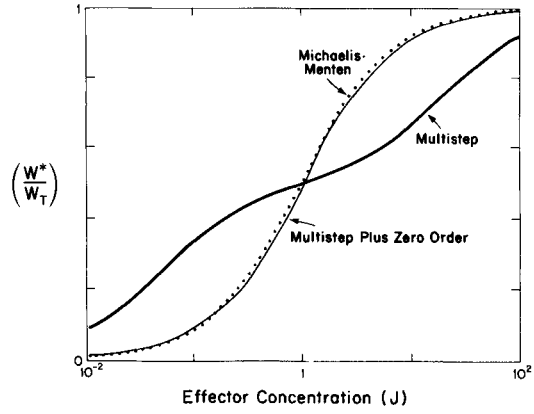

FIG. 8. **Interplay between multistep and zero-order effects in the monocyclic system.** An intermediary plateau due to multistep effect is suppressed by zero-order ultrasensitivity which renders the modification curve quasi-Michaelian. The curves are obtained as in Fig. 7, with the same parameter values except $K_{J1} = 0.1\ \mu M$, and $K_{J2} = 10\ \mu M$.

illustrated in Table I for $K_{m1} = K_{m2} = 10^{-1}\ W_T$. The values of V_1/V_2 at 10 and 90% W^* give an R_V of 3.24 which relates to the zero-order effect alone. The multistep effect can be calculated from Table I by assuming that W_T is very small, which results in an R_J of 11.7 for the multistep effect alone. The combination of the two effects gives a combined R_J of 1.9, a highly ultrasensitive response equivalent to an allosteric protein with a Hill coefficient of 8.

Bicyclic Cascade—We return to the cascade considered in Fig. 5 and determine now the effect of zero-order ultrasensitivity when superimposed on the multistep effect. To allow comparison with *curve a* of Fig. 6, we take the same parameter values and consider the case where the Michaelis constants of the four enzymes of the cascade are small, compared to the total amounts W_T and Z_T of their protein substrates.

Fig. 9 shows that when the zero-order ultrasensitivity obtains in cycle 1 alone (*a*), in cycle 2 alone (*b*), or in both cycles (*c*), the transition in Z^* is sharper than with the multistep effect alone (*curve a* of Fig. 6). The latter is characterized by a R_J value of 13.5, whereas R_J drops to 6.4 in *a*, 2 in *b*, and 1.5 in *c*. These values are obtained for a case where the zero-order effect is not exceedingly favored, given that W_T and Z_T are only taken ten times larger than their related Michaelis constants (except K_{m1} which is equal to W_T). Increased sensitivity could be obtained with smaller values of the Michaelis constants.

An intriguing effect can be obtained as a result of the interplay between zero-order ultrasensitivity and multistep inputs of the effector when the inhibition constant of J for E_2 is well below its inhibition constant for E_3. If constants are selected so that full transformation of W into the active form of W^* in the first cycle does not produce full transformation of Z into Z^* in the second cycle, a "staircase" modification curve can be obtained for Z^* as a function of the effector J (Fig. 10). This occurs when zero-order ultrasensitivity obtains in both modification cycles. The first sharp transition corresponds to the abrupt transformation of W into W^*, leading here to a transition in Z^* from less than 1% to close to 15%. Further increase in J does not change this situation much, until J exceeds the inhibition constant for

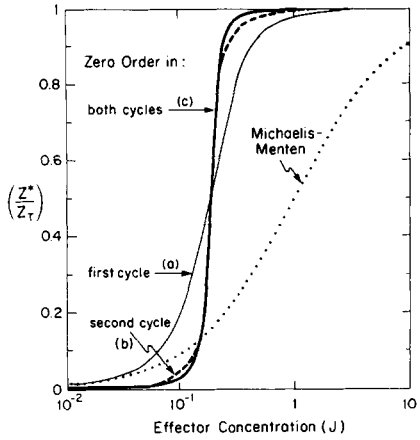

FIG. 9. **Interplay between zero-order and multistep ultrasensitivity in the bicyclic cascade of Fig. 5.** The multistep effect of Fig. 6 (*a*) is associated with zero-order ultrasensitivity in cycle 1 alone (*a*), in cycle 2 alone (*b*), or in both cycles 1 and 2 (*c*). Curves are obtained by using equations similar to Equations 2 or 10 for W^*/W_T and Z^*/Z_T, depending on whether zero-order ultrasensitivity is considered to occur or not. Parameter values are $(V_{M1}/V_{M2}) = 1$, $K_{J2} = K_{J3} = 2.10^{-8}$ M, $(K_{m1}/K_{m2}) = 10$, and $(K_{mW^*}/K_{m3}) = 1$, for all curves, with $K_{m2} = 0.1\ W_T$ and $K_{m3} = 0.1\ Z_T$ in the presence of a zero-order effect.

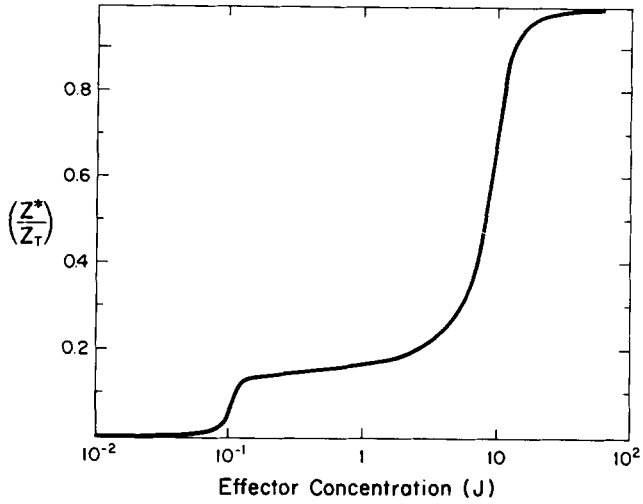

FIG. 10. **Staircase transition in a bicyclic modification cascade.** The effector J is a noncompetitive inhibitor of enzymes E_2 and E_3 in the cascade of Fig. 5. The modification curve is obtained as in Fig. 9 for the parameter values $(V_{M1}/V_{M2}) = 0.2$, $(V_{MW^*}/V_{M3}) = 0.6$, $(K_{m1}/K_{m2}) = 0.1$, $(K_{mW^*}/K_{m3}) = 0.1$, $K_{J2} = 0.03\ \mu$M, $K_{J3} = 20\ \mu$M, $K_{m2} = 0.1\ W_T$, and $K_{m3} = 0.1\ Z_T$.

E_3. Then, an increase in V_{W^*}/V_3, which was buffered until then, produces the second sharp transition from $Z^* = 0.15$ to $Z^* = 1$.

The possible significance of such staircase transitions lies in the cumulation of two ultrasensitivity responses associated with different levels of enzyme activity. Thus, in the case of Fig. 10, when expressing the inhibition constants in micromolar concentration, a variation in effector J from 0.08 to 0.11 μM could produce an increase in activity from 1 to 15% of V_{max}. Further increase in J would be buffered, until a change from $J = 3\ \mu$M to $J = 15\ \mu$M would result in an increase in activity from 20 to 90% of V_{max}. According to the need, a system could thus operate at quantized activity levels while preserving ultrasensitivity with respect to a controlling metabolite. Since it is obvious that the sharper the control the smaller the range over which control is exerted, this may be a way of preserving sharp transitions while maintaining control over a larger range.

DISCUSSION

The previous analyses of covalent modification systems have indicated that both the multistep effect and a zero-order effect can produce ultrasensitivity using parameters that are in the physiological range. Both are distinctly different from allosteric cooperativity but can combine with allosteric cooperativity and with each other to produce enhanced ultrasensitivity effects.

Multistep ultrasensitivity can arise from a single effector operating on different steps in a cascade. It has a theoretical limit based on the number of steps that the effector controls but it can approach close to that limit; for example, a single effector acting at two steps of the cascade will approach the ultrasensitivity of an allosteric dimer if all the parameters are in the optimal range. The conditions for optimal sensitivity are numerous, however, and if these parameters (the inhibition constants, the maximum enzyme activities, the Michaelis constants, etc.) are not optimal, the ultrasensitivity will be decreased dramatically and the response may in some cases be appreciably subsensitive. One of the conditions in which the multi-step effect is optimized occurs when the constant for activation of the forward step, for example, a kinase, is

much greater than the constant for inhibition of the reverse step, for example, a phosphatase. However, the reverse is not true, and only subsensitivity can be obtained when the dissociation constant for the activation step is far below the dissociation constant for the inhibition step. In principle, therefore, multistep ultrasensitivity can increase without limit if the number of steps controlled by the effector increases, but in practice it will be very unlikely if all the parameters can be in the optimal range.

Zero-order ultrasensitivity can therefore be far more effective than multistep ultrasensitivity, since it is possible to have an extremely dramatic step increase in activity in a single cycle. To obtain such a sizeable increase, however, again requires the values of all of the constants to be optimally related; moreover, the enzymes which are acted on must be saturating their converter enzymes. Obviously, in a multicycle cascade, this would put increasing demands on the concentration of enzymes. On the other hand, one does not need more than one or two cascades with appropriate constant to convert essentially 99% of an inactive enzyme to 99% in the active form. Zero-order ultrasensitivity therefore provides an effective switch mechanism which, because of the wide occurrence of protein covalent modification, could play a role in the control of a large number of cellular processes, both in normal and in pathological conditions.

In a multicycle cascade, the effector is not equally effective in any step. Thus, in a bicyclic cascade, for multistep control alone, it is far more efficient to exert regulation at the second of the two cycles. If the parameters for the second cycle are not optimal, the second cycle can severely dampen any ultrasensitivity generated in the first cycle.

An analysis of the interplay between the zero-order effect and the multistep effect shows that these two effects can act synergistically. In all cases this interplay tends to enhance the sensitivity gained for either effect alone. Zero-order ultrasensitivity tends to be the stronger of the two effects. When the multistep effect is subsensitive, the zero-order effect can even change the system to be at least as sensitive as a Michaelian system, and in some cases to become ultrasensitive. Either effect can of course give a further contribution to ultrasensitivity when the control of the converter enzymes, instead of being Michaelian, possesses some degree of cooperativity. Such is the case for the control of protein kinase by cyclic AMP (15).

The analysis suggests that zero-order ultrasensitivity can be distinguished from the multistep effect in any particular system simply by diluting the target protein from well above to well below the K_m of the modifying enzymes. When the converter enzymes are operating in the first-order region, there will be no zero-order effect and the only contribution will come from the multistep effect. The calculations show that increasing the enzyme concentration when keeping all other parameters constant should, under most circumstances, reveal the extent of the zero-order ultrasensitivity alone.

We have examined the time evolution of the modification system under first-order and zero-order conditions to make sure that the system can shift to a new steady state in times that are physiologically reasonable. The results clearly show that quite rapid times can be obtained using values which are consistent with known kinase and phosphatase turnover numbers existing in the literature.

An interesting effect pertains to the existence of a plateau region in the modification curve when an effector controls more than one cycle in a cascade. Then, as the effector J is varied, one can have two regions of zero-order ultrasensitivity separated by an intermediate region in which the system is practically insensitive to regulation by the effector J. The global sensitivity in going from 10 to 90% activity is therefore very low, but the steepness of the rise from 1 to 15% can be very abrupt, followed by a long plateau region and then resuming by a second abrupt rise in activity in a highly sensitive manner at some increased value of effector concentration. Whether this staircase or quantized effect exists in actual systems controlled by covalent modifications remains to be seen but it could be biologically effective, and previous predictions of such unusual plateau regions by similar theoretical studies for allosteric proteins (12, 13) turned out to have physiological counterparts. The existence of step transitions due to the zero-order effect means increased sensitivity but also a reduced range of control for a given effector. The staircase effect could provide both ultrasensitivity in response and the possibility of an extended range of control.

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