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Reversible enzyme-catalysed reactions: the quasi steady state as a quasi-equilibrium, and a correction to Haldane's kinetic constants and to secondary relationships involving kinetic constants.

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Abstract

For reversible enzyme-catalysed reactions obeying Henri-Michaelis-Menten kinetics, theoretical solution of the rate equations for the enzyme-substrate intermediate are generally incorrect when the quasi-steady state approximation, equating the rate of change of the concentration of the enzyme-substrate intermediate to zero, is used. For the simplest kinetic model used by Haldane, such a procedure does not reveal that in one direction, that starting with the reactant having the lower binding constant, the quasi-steady state is one of quasi-equilibrium, and Haldane's structure of the K_m written in terms of rate constants is incorrect. This is probably also true of more complex mechanisms in which the structure of k_{cat} may also be in error. Modern methods of numerical integration for the solution of rate equations, if applied to reversible reactions to obtain rate constants from measured catalytic constants, will require the correct expressions for k_{cat} and K_m . Furthermore, the (now called) Haldane relationship, relating the kinetic constants k_{cat} and K_m for the forward and reverse reactions to the equilibrium constant of a reaction, is now seen to be generally incorrect, and in addition another exception for a the theoretical validation of k_{cat}/K_m as a specificity constant arises.

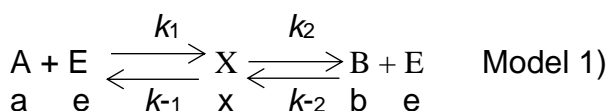
Key words

enzyme catalysis; Michaelis constant; kinetic constant; errors in k_{cat} ; errors in K_m ; quasi-equilibrium state; quasi-steady state; numerical integration

Introduction

An equation derived by Henri [1] and by Michaelis and Menten [2] describes the kinetics of many enzyme-catalysed reactions. Their derivations were based on reaction model 1, in which A and B are reactants, E the enzyme and X a reactant-enzyme intermediate, and the small case letters represent their respective concentrations at any instant. In modern form, the equation is written as equation 1 (e_0 is the total enzyme concentration, $e + x$), and it contains two kinetic constants: the catalytic constant, k_{cat} , and the Michaelis constant, K_m , which were originally defined in terms of rate constants (letters k). Originally, it was assumed that early in the reaction the reverse reaction could be neglected. This equation has one immediate solution for x (the equilibrium state of all components, readily shown by substitution), but otherwise, except by approximation, it has no analytical solutions. The approximation commonly used today was introduced by Briggs and Haldane [3] for an irreversible reaction: dx/dt is equated to zero, and the approximation is the now called quasi-steady state assumption. This approximation was later used by Haldane to solve the rate equations for both directions of reversible reactions and to define the kinetic constants pertinent to each direction [4], but his solutions were incorrect.

$$db/dt = k_{cat} ae_0 / (a + K_m) \quad (1)$$



$$dx/dt = (k_1a + k_{-2}b)e - (k_{-1} + k_2)x \quad (2)$$

Notwithstanding the early determinations of k_{cat} and K_{m} in terms of rate constants, as more complex kinetic models were examined, particularly for multi-substrate reactions, the common view was adopted around the middle of the last Century that the experimentally derived constants should be viewed as purely empirical ones. This may change as computer aided numerical methods are now used to determine with greater accuracy k_{cat} and K_{m} from primary data (concentrations measured at intervals rather than from secondarily derived velocities) [5]. Furthermore, methods of numerical integration [6,7] allow assessments of the best set of fluxes through, or rate constants pertaining to, each stage of the reaction models considered. These developments for the back conversion of experimental kinetic constants to sets of rate constants require the correct kinetic model and the correct kinetic constants pertaining to that model. Evidence that Haldane's derivations for reversible reactions (models 1 and 2, [4]) are defective appeared in the literature between 1958 and 1963 [8,9,10,11]. Haldane's derivations are correct only for the reaction in one direction, that starting with the reactant having the higher binding constant.

There are other consequences of the error resulting from the inadequacy of Haldane's analyses. The relationship, relating k_{cat} and K_{m} of the forward and reverse reactions to the equilibrium constant of the reaction, the now called Haldane relationship, is generally incorrect, and the use of $k_{\text{cat}}/K_{\text{m}}$ as an enzyme specificity constant receives a further example for which it is invalid.

In the following I shall restate the evidence for the incompleteness of Haldane's analyses, and provide for model 1 a theoretical proof that, if a starting reactant has a binding constant less than that of its product (and if the reaction does then indeed follow Henri-Michaelis-Menten kinetics) the quasi-steady state is one of quasi-equilibrium, a result not anticipated

by Haldane. I shall restrict my observations to the condition, usual for the characterization of an enzyme, that the reactant concentration is several orders of magnitude greater than that of the enzyme, a condition which may not always be filled *in vivo* or where enzymes are used *in vitro*.

RESULTS

The correct derivation of the Henri-Michaelis-Menten equation for Model 1.

Haldane's analysis of model 1 for an initial velocity (v_i), gave equation 3a for a reaction beginning with A, and 3b for that beginning with B (a_0 and b_0 are initial concentrations). The capital superscripts identify the reactant. These equations identify the kinetic constants in terms of rate constants:

$$K_m^A = (k_{-1} + k_2)/k_1, k_{cat}^A = k_2; K_m^B = (k_{-1} + k_2)/k_{-2}, k_{cat}^B = k_{-1}.$$

These results are well known, but **both** of these Michaelis constants are correct only when $k_1 = k_{-2}$. The K_m for the forward and reverse reaction are then identical, and the quasi-steady state is one of a quasi-equilibrium of X approaching its ultimate equilibrium concentration.

$$v_i^A = db/dt = k_2 a_0 e_0 / \{a_0 + (k_{-1} + k_2)/k_1\} \quad (3a)$$

$$v_i^B = da/dt = k_{-1} b_0 e_0 / \{b_0 + (k_{-1} + k_2)/k_{-2}\} \quad (3b)$$

For an irreversible reaction, x must proceed through a maximum value (when dx/dt is zero) before both x and dx/dt return to zero. For a reversible reaction, experimental evidence for the rôle of quasi-equilibrium (x_{equ}) in the steady state of X was produced about thirty years after Haldane's first use of the steady state approximation. Morales and co-authors [8,9] showed that, for model 1, x could pass through a maximum value greater than its equilibrium

concentration only if, in a reaction starting with A, $k_1 > k_{-2}$ (at the maximum $dx/dt = 0$ and d^2x/dt^2 is negative). Miller and Alberty [10] observed that there is an exact solution for equation 2 when $k_1 = k_{-2}$ (which occurs without the approximation that $a_0 \gg e_0$), and in this case x rises monotonically by an exponential function to its final equilibrium value, x_{equ} . The only quasi-steady state concentration of x is the one approaching x_{equ} . Using reasonable kinetic data, Miller and Alberty showed that x could rise very rapidly to values close to x_{equ} , and this was the first example of a quasi-steady state being one of quasi-equilibrium, but these authors did not point that out. They extended their observations by numerical approximation to the condition that $k_1 > k_{-2}$, and observed that x proceeded through a maximum before descending to equilibrium. They gave a single progress curve for the condition $k_{-2} = 10k_1$ in which x rose only to equilibrium. Walter and Morales [11] made computer-aided calculations of progress curves for x , and showed that when $k_1 = k_{-2}$ or $k_1 < k_{-2}$, x rises monotonically to x_{equ} , and more recently, Tzafiriri and Edelman [12] gave an example of x rising to x_{equ} when $k_1 < k_{-2}$. In appendix 1, I show by a kinetic analysis that, in a reaction starting with A and when $k_1 < k_{-2}$, it is to be expected that x rises monotonically to x_{equ} .

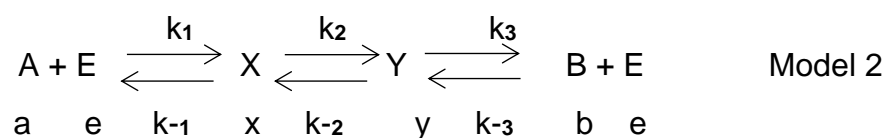
If the only steady state available for model 1 (in a reaction starting with A and with $k_1 = k_{-2}$ or $k_1 < k_{-2}$) is one in which x is approaching x_{equ} , then the initial quasi-steady state velocity would be written as $v_i = k_2 x_{\text{equ}}$. With $x_{\text{equ}} = a_0 e_0 / (a_0 + k_{-1}/k_1 + k_2/k_{-2})$ (appendix 2), this leads to equation 4 which gives the identities $k_{\text{cat}} = k_2$ and $K_m = (k_{-1}/k_1 + k_2/k_{-2})$. The K_m is not that derived by Haldane, except when $k_1 = k_{-2}$.

$$v_i = k_2 a_0 e_0 / (a_0 + k_{-1}/k_1 + k_2/k_{-2}) \quad (4)$$

It should be noted that, if the ratio k_{-2}/k_1 is too great or the equilibrium constant, ($K_{\text{equ}} = b_{\text{equ}}/a_{\text{equ}}$) is too small, Henri-Michaelis-Menten kinetics may not be observed. This is because x_{equ} is approached too slowly so that there is not a steady state such that, in the words of Briggs and Haldane [3] "...x is always negligible compared with b and a, its rate of change must, *except during the first instant of the reaction*, be negligible compared with theirs." (Emphasis added, but otherwise this quotation is altered only to identifying the concentrations of species with the abbreviations used here). The calculations of Walter and Morales [11] demonstrate this problem, at least with respect to the effect of decreasing K_{equ} . They presented progress curves of X for reactions in which k_1 , k_{-1} and k_2 were constant, and only k_{-2} was varied. As k_{-2} increases above k_1 , the slower approach of x to x_{equ} is clearly seen in their computations. Hence reversible reactions must be considered individually in terms of the ratio k_1/k_{-2} and K_{equ} . For initial velocities, they may display Henri-Michaelis-Menten kinetics in the direction which starts with the reactant with the higher binding constant (and have a K_m as derived by Haldane), and in the reverse direction provide a K_m which involves a quasi-equilibrium state. But if x_{equ} is approached too slowly it may not be possible to determine an initial velocity (in the usual sense that "initial" is used (see for example the calculation in [10]), and at which the product concentration may be considered negligible compared with the initial reactant concentration).

The correct derivation of the Henri-Michaelis-Menten equation for model 2.

Model 2 was introduced by Haldane [4] on the basis of experimental observations, and it



improved model 1 by allowing an isomerization of the intermediate X to Y before a product is released from Y. Although both dx/dt and dy/dt cannot in general be exactly zero at the same instant [11], the analytical methods of Morales and co-authors [8,9] can be applied to this model. The rate equations for x and y are given by equations 5 and 6, and their sum is equation 7a, which on substituting $a = (a_0 - b)$ becomes equation 7b. If y does proceed through a quasi- steady state maximum, when dy/dt is exactly zero and d^2y/dt^2 is negative, then dx/dt must also be negligible even if not exactly zero. (That statement might not be true if dx/dt and dy/dt were oscillating out of phase with each other, but in a closed system without a feed-back loop, the possibility of oscillation may be neglected). The second differential of y with respect to t is then given by equation 8. If dx/dt and dy/dt are negligible and zero, respectively, d^2y/dt^2 can be negative only when $k_1 > k_{-3}$. When this inequality is not obeyed, dy/dt can only be positive, becoming zero only at equilibrium.

$$dx/dt = k_1a(e_0 - x - y) + k_{-2}y - (k_{-1} + k_2)x \quad (5)$$

$$dy/dt = k_{-3}b(e_0 - x - y) + k_2x - (k_{-2} + k_3)y \quad (6)$$

$$dx/dt + dy/dt = (k_1a + k_{-3}b)(e_0 - x - y) - k_1x - k_3y \quad (7a)$$

$$dx/dt + dy/dt = \{k_1a_0 + (k_{-3} - k_1)b\}(e_0 - x - y) - k_1x - k_3y \quad (7b)$$

$$d^2y/dt^2 = (k_{-3} - k_1)(e_0 - x - y)db/dt - \{k_1a_0 + (k_{-3} - k_1)b + k_3\} dy/dt - \{k_1a_0 + (k_{-3} - k_1)b + k_3\} dx/dt \quad (8)$$

A general evaluation of d^2y/dt^2 cannot be made as I have done for d^2x/dt^2 in model 1 (appendix 1) because the relationships between x and y, and dx/dt and dy/dt , are not known except in a particular special case, that when the rate constants for the interconversion of X and Y (k_2 and k_{-2}) are large enough to keep X and Y in equilibrium with each other. Then,

$k_2x = k_{-2}y$, $k_2 dx/dt = k_{-2}dy/dt$, and this allows $(x+y)$ to be treated as a single unit. Of course, this reduces model 2 to model 1. It is sufficient here to note that, when a reaction starts with A and $k_{-3} > k_1$, k_{cat}^A remains that given by Haldane, but K_m^A does not.

In view of what has been noted so far about model 1 and the special case of model 2, it seems likely that for model 2 there would be a general problem with Haldane's derivations of the kinetic constants. This view is supported by comparing Haldane's result [4] for the steady state concentration of Y (y^* in equation 9) with its equilibrium concentration (y_{equ}), (equation 10, see appendix 2). Equation 9 derives from Haldane's general equation [4] when the concentration b is set to zero. Expanding the inequality $y^* > y_{equ}$ and cancellation leads to inequality 11. The right hand side of inequality 11 must be positive, which leads to inequality 12 as a requirement for y^* to be greater than y_{equ} . Once again, if there is not a maximum value of y (before equilibrium), y can only rise to its equilibrium value. This observation, however, does not prove that y rises monotonically so that, although dy/dt is always positive (zero at equilibrium), d^2y/dt^2 is always negative (zero at equilibrium).

$$y^* = k_1 k_2 a_0 e_0 / \{k_1 a_0 (k_{-2} + k_2 + k_3) + k_{-2} k_{-1} + k_{-1} k_3 + k_2 k_3\} \quad (9)$$

$$y_{equ} = k_1 k_2 a_0 e_0 / \{k_1 a_0 (k_{-2} + k_2) + k_{-1} k_{-2} + (k_1 k_2 k_3 / k_{-3})\} \quad (10)$$

$$k_1 a_0 < k_2 (k_1 / k_{-3} - 1) - k_{-1} \quad (11)$$

$$k_1 / k_{-3} > 1 + k_{-1} / k_2 \quad (12)$$

Consequences of a misidentified Kinetic Constants.

i. The Haldane Relationship. Haldane derived his relationship (equation 13) for both models 1 and 2 [4], but these derivations depended on his identification of the catalytic constants in terms of rate constants. For model 1 and the conditions that $k_{-2} > k_1$, these are $k_{cat}^A = k_2$ and $K_m^A = (k_{-1}/k_1 + k_2/k_{-2})$, but for the reverse reaction k_{cat}^B and K_m^B will then be k_{-1} and $(k_{-1} + k_2)/k_{-2}$, respectively. Substitution of these in equation 13 does not give $K_{equ} = k_1 k_2 / k_{-1} k_{-2}$. Equation 13 is valid only when $k_1 = k_{-2}$, when in both directions the quasi-steady states involve quasi-equilibrium [10]. A similar conclusion may be adduced for model 2 when $k_1 = k_{-3}$ and k_2 and k_{-2} maintain equilibrium between X and Y.

$$K_{equ} = (k_{cat}^A / K_m^A) / (k_{cat}^B / K_m^B) \quad (13)$$

It may be noted that Bock and Alberty [13] made well-controlled kinetic measurements using fumarase, and claimed to have validated the Haldane relationship. Although the reaction catalysed by the enzyme is not one with a single reactant and product in both directions, in the conversion of fumarate to malate the concentration of water can be considered a constant and combined with a second order rate constant. Since the original work, however, fumarase has been shown to be a tetramer with four identical subunits, and extensive kinetic studies by Rose [14] have shown there are several possible kinetic pathways within each subunit. Mescam et al. [6] determined the relative fluxes through three possible kinetic pathways of an 11-state reversible mechanism which they adapted from the work of Rose. Their results indicate that, except for the binding steps, each pathway had intermediate stages in a state of quasi-equilibrium. This would lead to the prediction that Henri-Michaelis-Menten-kinetics would be displayed by each pathway separately (but the summation for the two such pathways does not do so unless their Michaelis constants were identical). The actual mechanism of fumarase is certainly not that of either model 1 or 2, and so the results of Bock and Alberty [13] are not pertinent to the Haldane relationship.

ii. Enzyme specificity. The ratio k_{cat} / K_m is perhaps best known as a measure of enzyme efficiency and specificity. This may be seen intuitively from a consideration of equation 1: when $a_0 \ll K_m$, db/dt approximates to $a_0 e_0 k_{cat}/K_m$, and the velocity is maximized for the lowest reactant concentration. It has been shown that k_{cat}/K_m is not always useful for comparing different enzymes catalysing the reaction of a single substrate [15], and unfortunately, for the case when the reactions of two substrates are catalysed in a single solution by one enzyme, it must now be stated that k_{cat}/K_m is also not always a good measure of specificity. This latter approach to specificity was introduced Fersht [16], and Cornish-Bowden [17], and Cornish-Bowden [18] also extended the use of k_{cat}/K_m to reactions more complex than model 1. Unfortunately, none of these avoided the problem which I have pointed out above, the difficulty of simply equating dx/dt to zero in a rate equation and following Haldane to expressions for k_{cat} and K_m . I shall not develop proofs here because for model 1 they are elementary, but simply state that if two competing substrates both have binding constants greater than those of their respective products, then the analyses of Fersht and Cornish-Bowden are valid. This is also true in the case when the steady state of both competing reactants involves a quasi-equilibrium, but should the steady state for one reactant be as described by Haldane and for the other a quasi-equilibrium, then the analysis is not correct.

Discussion

When Haldane [4] applied the steady state approximation of Briggs and Haldane [3] to reversible reactions, he did not consider the possibility that the steady state in model 1 could be one in which x was close to and approaching its final equilibrium concentration, a possibility first revealed almost thirty years later [8,9,10,11]. In fairness to Haldane, it should be noted that he may not have been aware of the work of Morales and co-authors [8,9],

Miller and Alberty [10], and Walter and Morales [11]. He wrote an Introduction to the second edition of his book [19] in 1964, the year of his death in India at the age of 72, and made clear in the interesting introduction to that edition why his book was deliberately republished unchanged.

I have no explanation for the fact that the consequences resulting from the observations of Morales and co-workers, of Miller and Alberty, and of Walter and Morales have been overlooked, and certainly the study of enzyme kinetics has moved on without them. None the less, Haldane's derivations of kinetic constants for models 1 and 2 are still quoted without question, and there are numerous publications, in which the solution of rate equations of more complex reaction models, are obtained unquestioningly by simply equating dx/dt to zero. In all probability, for reversible reactions these would be incorrect in one direction. Haldane's relationship too is, in general, incorrect.

The justifications for the use of k_{cat}/K_m as a measure of specificity also appear in text books and in published work, and appears to be standard material in texts on enzyme kinetics published or re-published in this Century. As I have pointed out above, in the context of two reactants in solution with a single enzyme, it is valid only if for both reactions the quasi-steady state kinetic parameters are as determined by Haldane, or both involve quasi-equilibria, but this cannot be established without knowledge of the binding constants for both directions of each reaction. I am not aware of a case in which these have been determined.

A recent publication by Johnson [7], has revealed an interesting property of the ratio k_{cat}/K_m , although it is to be hoped that the name of specificity constant might be replaced. After the phase of referring to k_{cat} and K_m as purely empirical constants fitting the Henri-Michaelis-

Menten equation, it is now possible using methods of numerical integration to determine the best set of rate constants which may fit a theoretically derived equation (for k_{cat} and K_m) to its experimentally measured value. Should this approach be extended to reversible reactions, this will require the correct deduction of the kinetic constant in terms of rate constants, for which only a start has been made here.

Appendix 1. The steady state in model 1 with a reaction starting with A and $k_1 < k_{-2}$.

The quasi-steady state approximation of Briggs and Haldane [3] stated, "...since x is always negligible compared with b and a, its rate of change must, except during the first instant of the reaction, be negligible compared with theirs." (This quotation is altered only to identifying the concentrations of species with the abbreviations used here). dx/dt must be negligible, but not necessarily zero, and when dx/dt is always positive (except at equilibrium) this condition could be met if dx/dt initially rose rapidly to a phase where it became negligible (and which could be a quasi-steady state), and after which it rose more rapidly before finally declining as x reached its equilibrium concentration. Such a process would require that the sign of d^2x/dt^2 is initially negative, passes through a positive phase and then a further negative one as the equilibrium concentration is reached. This scenario would lead, if dx/dt were approximated to zero, to the kinetic constants predicted by Haldane, but it cannot occur with model 1 when $k_{-2} > k_1$. Provided $a_0 \gg e_0$, d^2x/dt^2 is always negative (zero at equilibrium), there are no inflexions in the progress curve of X, and x rises monotonically to x_{equ} . This is demonstrated as follows.

The rate equation for model 1 is equation 2, and with $e = (e_0 - x)$ and $a = (a_0 - b)$ it is here rewritten here as i). Its partial differentiation gives equation ii) in which the term multiplying dx/dt , $\{k_1 a_0 + (k_{-2} - k_1) b + k_{-1} + k_2\}$, is always positive, and dividing equation ii) by

this term (which is now written as N) will not alter the sign of d^2x/dt^2 . Inserting the rate equations for db/dt and dx/dt , this leads to equation iii). d^2x/dt^2 will be negative if

$$\begin{aligned} & [(e_0 - x)\{k_1a_0 + (k_{-2} - k_1) b\} - x(k_{-1} + k_2)] \\ & > (k_{-2} - k_1)(e_0 - x)\{k_2x - k_{-2}b(e_0 - x)\}/N, \end{aligned}$$

Dividing both sides of the inequality by $(e_0 - x)$, which is positive, and rearrangement leads to the requirement iv). Provided that $a_0 \gg e_0$, the terms $(k_{-2} - k_1)(e_0 - x)k_{-2}b/N$ can be neglected in comparison with $\{k_1a_0 + (k_{-2} - k_1) b\}$, and $(k_{-2} - k_1)(e_0 - x)k_2x/N$ can be neglected compared with $(k_{-1} + k_2)x/(e_0 - x)$, leading to equation v). This equation is $dx/dt / (e_0 - x)$, and this is always positive when $k_{-2} > k_1$ and dx/dt has no negative phase. Hence d^2x/dt^2 is always negative, and x rises monotonically to x_{equ} .

$$dx/dt = (e_0 - x)\{k_1a_0 + (k_{-2} - k_1)b\} - x(k_{-1} + k_2) \quad \text{i)}$$

$$d^2x/dt^2 = (k_{-2} - k_1)(e_0 - x) db/dt - dx/dt\{k_1a_0 + (k_{-2} - k_1) b + k_{-1} + k_2\} \quad \text{ii)}$$

$$\begin{aligned} d^2x/dt^2/N = (k_{-2} - k_1)(e_0 - x) \{k_2x - k_{-2}b(e_0 - x)\}/N \\ - (e_0 - x)(k_1a_0 + (k_{-2} - k_1) b) - x(k_{-1} + k_2) \quad \text{iii)} \end{aligned}$$

$$\{k_1a_0 + (k_{-2} - k_1) b\} + (k_{-2} - k_1)(e_0 - x)k_{-2}b/N >$$

$$\{(k_{-2} - k_1) k_2x/N - (k_{-1} + k_2)\}x/(e_0 - x) \quad \text{iv)}$$

$$\{k_1a_0 + (k_{-2} - k_1) b\} - (k_{-1} + k_2)x/(e_0 - x) > 0 \quad \text{v)}$$

Appendix 2. The equilibrium concentration of enzyme-reactant intermediates.

The equation $x_{\text{equ}} = a_0e_0 / (a_0 + k_{-1}/k_1 + k_2/k_{-2})$ is stated in reference 10 . It is obtained using the same principles as outlined here for model 2. There are two conservation equation

for model 2: $e_o = e + x + y$, and (when $a_o \gg e_o$) $a_o = a + b$. Where the small case letter (except a_o and e_o) are now understood to represent equilibrium concentrations, there are the following equilibrium conditions.

$$b/a = K_{\text{equ}} = k_1 k_2 / k_{-1} k_{-2}, \quad x = k_{-2} y / k_2, \quad e = k_3 y / k_{-3} b,$$

$$b = a K_{\text{equ}} = (a_o - b) K_{\text{equ}} \quad \text{so that } b = a_o K_{\text{equ}} / (1 + K_{\text{equ}}),$$

$$\text{and } e = k_3 y (1 + K_{\text{equ}}) / k_{-3} a_o K_{\text{equ}}$$

Hence, $e_o = y \{1 + k_{-2}/k_2 + k_3(1 + K_{\text{equ}}) / k_{-3} a_o K_{\text{equ}}\}$, from which with substitution for K_{equ} and rearrangement one arrives at

$$y_{\text{equ}} = k_1 k_2 a_o e_o / \{k_1 a_o (k_{-2} + k_2) + k_{-1} k_{-2} + (k_1 k_2 k_3 / k_{-3})\} \quad (\text{equation 10}).$$

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